Blood Parameter Responses in Mice (*Mus musculus*) Exposed to an Electromagnetic Field

by

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Declaration

I, Henry Charles Leighton Gleimius, identity number [redacted] and student number [redacted], do hereby declare that this research project submitted to the Central University of Technology, Free State, for the Degree of Doctor of Philosophiae in Environmental Health, is my own independent work. This work complies with the code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any other institution by myself or any other person for the attainment of a qualification.

Henry Charles Leighton Gleimius

2017

I certify that the above statement is correct.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ALL	acute lymphocytic leukaemia	MHz	megahertz
A/m	ampere per meter	mRNA	Messenger Ribonucleic acid
Ca^2	calcium ion	MCV	mean corpuscular volume
CD	cluster of differentiation	MPV	mean platelet volume
cm	centimetre	m	metre
mm^3	cubic millimetre	µm	micrometre
DNA	deoxyribonucleic acid	µT	microtesla
E	photon energy	mT	millitesla
ELF-EMF	extremely low frequency electromagnetic field	mG	milligausss
EHz	exahertz	nm	nanometre
Exp.	experiment	PHz	petahertz
F	frequency	pm	picometre
G	gauss	PLT	platelet
GHz	gigahertz	PVC	polyvinyl chloride
GPS	global positioning system	RBC	red blood cell
HGB	haemoglobin	RNA	ribonucleic acid
HCT	haematocrit	THz	terahertz
H	hour	T	tesla
h/d	hours per day	VGCC	voltage-gated Ca^{2+} channels
Hz	hertz	V/m	volt per meter
IARC	International Agency for Research on Cancer	λ	wavelength
kHz	hilohertz	WBC	white blood cell
km	kilometre	WHO	World Health Organisation

Abstract

Introduction: Extremely low frequencies of electromagnetic radiation are omnipresent where humans reside. These electromagnetic radiations are in the form of electrical power lines, transformers, electric hardware, and many household devices, such as hairdryers, electrical blankets and devices used in medicine and industry. The biological effects of extremely low frequencies of electrometric fields (ELF-EMF) at the cellular level have been linked with mice blood and immunological parameters. An understanding of cellular biological effects of ELF-EMF, particularly in the long term, is still lacking. The aim of this study thus was to measure the effects of ELF-EMF on 15 blood parameters in 4-week old healthy male mice (*Mus musculus*); of the BALB/c strain. Blood parameters included the number of erythrocytes, leucocytes and four lymphocyte cluster of differentiation markers, and other related parameters. The expected outcome of the study is to show that ELF-EMF has an effect on blood parameters of mice. The two research questions pertaining to this study were: 1. To what extent does one-week exposure to ELF-EMF influence blood parameters in the BALB/c N1H mouse strain? 2. To what extent does twelve-week exposure to ELF-EMF influence blood parameters in the BALB/c N1H mouse strain?

Methods: Two ELF-EMF exposure regimes were applied; a short-term exposure of one week (Experimental Trial 1) and a longer-term exposure of 12 weeks (Experimental Trial 2). Besides the control treatment group, a one-hour, four hours and 24-hours exposure treatments were applied daily to 25 randomly selected mice in each treatment group, at a magnetic flux density of 5 µT. The mice were housed in specially designed polypropylene mouse cages. At the end of the two trials, blood was collected from the orbital cavities of the mice for analyses.

Results and discussion: In Experimental Trial 1, ELF-EMF did not have a significant ($p > 0.05$) effect on the red blood cell population and the related parameters haemoglobin concentration and haematocrit; as well as on the number of thrombocytes and total leucocytes. The different leucocyte cell types did, however, show significant changes ($p < 0.0001$). The number of granulocytes,

eosinophils and basophils, was elevated, but the number of neutrophils was suppressed. The T-lymphocyte CD3, CD4 and CD8 populations demonstrated significant increases in number, particularly in the one-hour ELF-EMF exposure group ($p < 0.001$). The B-lymphocyte CD19 populations, did not show any differences when compared to the control treatment group ($p = 0.57$). When analysing the results of Experimental Trial 2, it was found that the control treatment groups were, for the most, highly significantly different when compared to the short-term exposure experiment ($p < 0.001$). These data indicated that long-term confinement resulted in other physiological changes, irrespective of whether the animals were exposed to ELF-EMF or not. These changes could probably be attributed to subclinical stress conditions brought about by confinement and the age of the animals. The control treatment groups showed suppressed numbers of total leucocytes, lymphocytes, monocytes, neutrophils and basophils, while the eosinophils were significantly elevated when compared to the short-term experimental control groups. The control groups of the T-lymphocyte CD3, CD4 and CD8 populations were also elevated when compared to the short-term experiment. In Experimental Trial 2, no significant differences could be established between the control treatment groups and the ELF-EMF exposure groups in all the parameters. It appears as if ELF-EMF adaptation and subclinical stress conditions modified the numbers of the respective parameters to such an extent that the control treatment and exposure treatment groups presented with equivalent numbers, making it difficult to isolate the effect of ELF-EMF exposure. Although the control treatment and treatment groups of the different parameters did not differ from one another, it appeared as if the four-hour ELF-EMF exposure may have contributed to the elevation of the number of red blood cells, haemoglobin concentration and haematocrit. Similarly, the basophils were also elevated in the one-hour ELF-EMF exposure group.

Conclusions: The unexpected results of this study revealed that many gaps still exist in the understanding of the underlying effects of ELF-EMF exposure on living tissue, particularly blood parameters. How the immune response is activated and the role of confinement of animals and its

effect on the results of experiments needs further investigation. Finally, how the effects of ELF-EMF exposure in mice relate to ELF-EMF exposure in humans, also requires further elucidation.

Chapter 1

Introduction

1.1 Introduction

The role and effects of electromagnetic fields in the lives of living organisms have interested scientists for more than two centuries. A hundred years ago, Ernest Solvay (1838–1922), a Belgian chemist, noted that "The phenomenon of life can and should be explained by the action of only physical forces which govern the universe and that, among these forces, electricity plays a predominant role" (Bassett 1992). The recognition of electromagnetism; the unity of electric and magnetic phenomena, is largely due to the contributions of Hans Christian Ørsted and André-Marie Ampère in 1819–1820 (Manzetti and Johansson 2012).

Humans are constantly exposed to a variety of sources of electromagnetic radiation. Natural sources of electromagnetic radiation include sunlight, cosmic rays, and terrestrial radiations. However, since early in the 20th century exposure to artificial sources of electromagnetic radiation has increased substantially (Calvente et al. 2010). These sources include power stations, radio, radar, television, computers, mobile phones, microwave ovens, and numerous devices used in medicine and industry.

Extremely low frequencies of electromagnetic radiation are omnipresent where humans reside. These electromagnetic radiations are in the form of, for example, electrical power lines, transformers, alternators, electric hardware, equipment of arc- and resistance-soldering and many household devices, such as hairdryers, electrical blankets and ovens (Touitou et al. 2013; Redlarski et al. 2015). In many cases pollution by extremely low frequencies of electromagnetic radiation is much stronger than any natural sources of electromagnetic fields or radiation (Redlarski et al. 2015).

Advances in electromagnetic radiating technologies have aroused concerns about the potential health risks associated with exposure (Calvente et al. 2010). There has been considerable concern about the potential effects associated with extremely low frequency electromagnetic fields (ELF-EMF) on the health of human populations (Patruno et al. 2015). Many epidemiologic studies have shown an association between ELF-EMF and cancer (Touitou et al. 2013; Redlarski et al. 2015). One of the first scientific studies supporting the notion that ELF-EMF are harmful to humans was demonstrated by Nancy Wertheimer and Ed Leeper in 1979 (Manzetti and Johansson 2012). These authors were looking for possible causes for a number of childhood leukaemia cases in the Denver metropolitan area. The outcome of this study showed that children with leukaemia were more than twice as likely to have lived in homes near high current power lines, where the electromagnetic fields were stronger. Further studies in the United States of America showed a statistical correlation between the emergence of childhood leukaemia mortality (at age 2–4 years) in the 1930s and the spread of residential electrification (Manzetti and Johansson 2012). Several epidemiological studies have linked ELF-EMF with an increased risk of cancer, for instance childhood leukaemia, brain cancer, breast cancer, kidney cancer, cancer of the nervous system and lymphoma (Patruno et al. 2015). Association of ELF-EMF with other diseases include cardiovascular diseases, depression, suicide, and neurodegenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis (Touitou et al. 2013; Patruno et al. 2015).

The biological effects of ELF-EMF at the cellular level have been noted in numerous studies. Particularly, studies on mice have indicated a link between ELF-EMF blood and immunological parameters (Dasdag et al. 2002). However, despite the volume of studies available, an understanding of the biological effects at the cellular level is still lacking; particularly the effects of long-term exposures (Kavet et al. 2001; Foster 2003; Santini et al. 2009; Patruno et al. 2015).

Animal models are extensively used to provide insight into the mechanisms underlying many human diseases. Although larger species, such non-human primates, are more closely related to humans; working with these large animals is extremely expensive and is fraught with ethical concerns. Thus, are thus extensively used. Mouse models are suitable for medical testing because their genetic, biological and behaviour characteristics closely resemble those of humans, and many symptoms of human conditions can be replicated in mice. To obtain insight into the effects of ELF-EMF on human blood parameters, a mouse model (BALB/c N_1H mouse strain) was subjected to ELF-EMF exposure. Therefore, the aim of this study was to measure the effects of an ELF-EMF on blood parameters of mice (*Mus musculus*). Two exposure regimes were applied, namely, a short-term exposure of one week and a longer-term exposure of 12 weeks. The blood parameters that were measured included the number of erythrocyte, thrombocyte, as well as a number of and leucocyte parameters. Four cluster of differentiation antigens (CD) on lymphocytes were also enumerated in this study. The expected outcome of the study is to show that ELF-EMF has an effect on blood parameters of mice.

The research questions pertaining to this study were:

1. To what extent does one-week exposure to ELF-EMF influence blood parameters in the BALB/c N_1H mouse strain?
2. To what extent does twelve-week exposure to ELF-EMF influence blood parameters in the BALB/c N_1H mouse strain?

To answer these research questions, the following objectives were devised:

I. To design and build a system in which mice can be exposed to different ELF-EMF treatment regimens;
II. To apply different expose treatment regimens of ELF-EMF to different groups of mice for shorter and longer periods;

III. To determine the effects of the different exposure treatment regimens of ELF-EMF on a number of blood parameters; and

IV. To analyse the data and reach conclusions.

1.2 Ethical consideration

The Animal Ethics Committee of the University of the Free State, Bloemfontein, Free State, South Africa, granted ethical approval for this project (see the Appendix for the ethics approval letter). This research project was executed in the animal house of the University of the Free State.

1.3 Layout of thesis

This thesis is arranged into seven chapters.

Chapter 1: Introduction

In this chapter, the field of study is introduced, the research problem identified, and the aim and objectives stated.

Chapter 2: Literature review

In this chapter, existing literature discussing the effects of ELF-EMF on human and animals is reviewed.

Chapter 3: Materials and methodology

In this chapter, a detailed description of the two experimental trials conducted in this study is provided, including the different exposure treatment regimens. A description of the construction of the experimental laboratories, as well as the methods used to determine the effects of ELF-EMF on the different mice blood parameters are also described.

Chapter 4: Erythrocytes and thrombocytes

In this chapter, the results of the effects of the different exposure treatment regimens of ELF-EMF on the mice erythrocyte parameters and thrombocytes are presented and discussed.

Chapter 5: Leucocytes

In this chapter, the results of the effects of the different exposure treatment regimens of ELF-EMF on the mice leucocyte parameters are presented and discussed.

Chapter 6: Cluster of differentiation

In this chapter, the results of the effects of the different exposure treatment regimens of ELF-EMF on the mice cluster of differentiation (CD) parameters are presented and discussed.

Chapter 7: Discussion and conclusions

In this concluding chapter, the key findings of this research project are discussed and conclusions presented. Potential future research is also recommended.

Appendix:

The appendix contains the ethical clearance letter from the Animal Ethics Committee of the University of the Free State, Bloemfontein, Free State, South Africa.

References:

The references in this document have been prepared using the reference manager Mendeley.

Chapter 2

Literature Review

2.1 Introduction

Exposure to electromagnetic fields is not a new phenomenon. During the twentieth century, exposure to electromagnetic fields started to increase steadily, particularly because of the ever-growing demand for electricity and ever-advancing technologies. Continuous changes in social behaviours have also created more and more artificial sources of electromagnetic fields. People have constantly been exposed to a complex mixture of weak electric and magnetic fields, arising from the generation and transmission of electricity by domestic appliances and industrial equipment. Electromagnetic fields influence the human body just as they influence any other material made up of charged particles.

2.2 Electromagnetic spectrum

2.2.1 History of the electromagnetic spectrum

Historically, visible light was the first component of the electromagnetic spectrum to be studied. The ancient Greeks recognised that light travelled in straight lines and later described the properties of reflection and refraction. Properties of the electromagnetic spectrum beyond visible light started to emerge in the 19th century, when William Herschel (1738–1822) identified electromagnetic radiation with the discovery of infrared radiation (Herschel 1800). He also noticed the temperature differences between colours of the colour spectrum. Later, Johann Ritter (1776–1810) discovered ultraviolet radiation, which lay beyond visible violet light rays of the electromagnetic spectrum (de Andrade Martins 2012). Michael Faraday (1791–1867) noticed that light became polarised when it passed through a transparent object in a magnetic field. At this time in 1845, he was able to link electromagnetism to electromagnetic radiation (de Andrade Martins 2012). A few years later, James

Maxwell (1831–1879) developed four partial differential equations for the electromagnetic spectrum. Two of the equations predicted the presence and behaviour of waves in the spectrum. Maxwell realised that these waves travelled at the speed of light and concluded that light was a type of electromagnetic wave (Maystre 2014). He also predicted waves of very low frequencies when compared to infrared. This was the first indication of the existence of the entire electromagnetic spectrum.

Later in the 19th century, radio waves and microwaves were described. The physicist Heinrich Hertz (1857–1894) built an apparatus that generated and detected radio waves. He also produced and measured microwaves (Maystre 2014). The discovery of radio waves and microwaves paved the way to the inventions of the wireless telegraph and the radio.

Wilhelm Röntgen (1845–1929) discovered a new type of radiation in 1895 called x-rays. He noticed that x-rays were able to travel through parts of the human body (Vetter 2016). These discoveries lead to the modern day use of x-rays in medical diagnostics and therapeutic procedures.

Early in the 20th century, Paul Villard (1860–1934) studied the radioactive emissions of radium. He described a type of radiation similar to known alpha and beta particles with a high level of penetration ability (Gerward and Rassat 2000). Later, William Henry Bragg (1862–1942) demonstrated that the alpha and beta particles described by Villard were electromagnetic radiation and not particles (del Regato 1981). Ernst Rutherford (1871–1937) named this type of radiation gamma rays in 1903 (del Regato 1981).

2.2.2 Range of the electromagnetic spectrum

The electromagnetic spectrum describes the range of all electromagnetic waves. Electromagnetic waves have both an electrical and a magnetic component (International Agency for Research on

Cancer 2002; Hawkes et al. 2014). Typically, electromagnetic waves can travel without a medium and thus makes it possible to see light from distant stars and planets because light can travel through the vacuum of space. At the one extreme of the electromagnetic spectrum are the waves with the lowest frequencies; while at the other extreme are the waves with the highest frequencies (IARC 2002; Hawkes et al. 2014).

The electromagnetic spectrum has been divided into regions defining each of the different wave types. Although this division is accurate, in reality there is often some overlap between neighbouring types of electromagnetic waves. At the low frequency end of the spectrum are, for example, radio and microwaves, with extremely low frequency waves lying at the extreme end of the low frequency end (Scenihr 2007). At the high frequency end of the electromagnetic spectrum are gamma rays with the highest frequencies of all the electromagnetic waves (Genuis 2008). Although the extreme ends of the electromagnetic spectrum have been defined, it is understood that the electromagnetic spectrum is continuous (Hawkes et al. 2014). The limit for long wavelengths is the size of the universe itself, while it is thought that the short wavelength limit is in the vicinity of the Planck constant (1.616×10^{-35} m) (Hawkes et al. 2014).

Electromagnetic waves are typically described by three physical properties. These properties are wavelength (λ), frequency (f) and photon energy (E) (Genuis 2008). Since all electromagnetic waves travel at the speed of light, the wavelength can be determined by dividing the speed of light by the wave's frequency (Table 2.1). Thus, wavelength is inversely proportional to the wave frequency. This means that as frequencies increase, the wavelengths decrease (Kostoff and Lau 2013). Photon energy is directly proportional to wave frequency. Extremely low frequency electromagnetic waves have low photon energies, while high frequency gamma rays have high photon energies (Genuis 2008).

Table 2.1 Frequencies, wavelengths and applications of electromagnetic waves.

Frequency zone	Frequency	Wavelength in air	Examples of applications	Author
Extremely low	< 300 Hz	> 1000 km	Many biological processes	Scenihr (2007)
Low, middle, high	30 kHz–30 MHz	10 km–10 m	Amateur radio, remote controls	Hitchcock (2012)
Very high, ultra-high	30–300 MHz	10–1 m	Radio and television	Hitchcock (2012)
Super high	300 MHz to 30 GHz	1 m–10 cm	Satellite communication, GPS	Hitchcock (2012)
Extreme high	30–300 GHz	10–1 cm	RADAR	Hitchcock (2012)
Infrared	300 GHz to 400 THz	1 mm–750 nm	Television remote	Calvente et al. (2010)
Visible light	400–790 THz	760–400 nm	Phototherapy	Calvente et al. (2010)
Ultraviolet	3 PHz–30 PHz	400–10 nm	Sterilisation in the medical field	Hawkes et al. (2014)
X-rays	30 EHz–300 PHz	1 nm–10 pm	Airport security and medical diagnostic imaging	Hawkes et al. (2014)
Gamma rays	300 EHz	> 1 pm	Medical diagnostic imaging	Hawkes et al. (2014)

Electromagnetic waves of the different parts of the electromagnetic spectrum interact in different ways with matter. Typically, if the wavelength of electromagnetic waves is of a similar size to that of a particular object, then it is possible to probe the object with that frequency of light (Hawkes et al. 2014). Gamma rays, x-rays and ultraviolet rays are classified as ionising electromagnetic waves (Calvente et al. 2010). These electromagnetic waves can cause damage to the human body, for example, by breaking down deoxyribonucleic acid (DNA) and causing cell damage (Calvente et al. 2010; Greinert et al. 2015). Ultra violet light also may cause skin cancer in humans and animals (Greinert et al. 2015). At the low frequency end of the electromagnetic spectrum, non-ionising waves

are found. These include visible light, microwaves, radio waves and, extremely low frequency electromagnetic waves (Nicolaou et al. 2011).

2.3 Extremely low frequency electromagnetic waves

Extremely low frequency electromagnetic waves are found at the low frequency end of the electromagnetic spectrum. Generally these waves fall in the category of frequencies < 300 Hz (Otto and von Mühlendahl 2007; Ortega-Garcia et al. 2009; Redlarski et al. 2015; Zhang et al. 2016) with wavelengths > 1,000 km (Kostoff and Lau 2013). These wave types surround all electrical wiring and appliances (Saunders 2003). Normally, the electric and magnetic fields occur together. So, when an electric current runs through an electrical wire or appliance an electromagnetic field is generated surrounding the wiring and appliance. Magnetic fields are not shielded by most common materials and easily pass through them (World Health Organisation 2007; Hawkes et al. 2014). However, when an appliance is switched off the magnetic field disappears and only the electric field remains (Saunders 2003; WHO 2007).Both the electric and magnetic fields weaken with increased distance from the source (Ortega-Garcia et al. 2009).

The electric and magnetic fields are measured using a variety of measuring units. The strength of the electric field is dependent on the voltage difference between two points in a circuit (WHO 2007). This voltage difference is measured in volts per metre (V/m) (WHO 2007). The magnetic field surrounding an electric current produces a flow of magnetic energy around the electric current, referred to as magnetic flux. Magnetic flux is a measure of the strength of a magnetic field around an electric current, based on the total number of magnetic lines of force that pass through a specific area (Hawkes et al. 2014). On the other hand, the magnetic flux density is the amount of magnetic flux in a unit area perpendicular to the direction of magnetic flow. Magnetic flux density is measured in Tesla (T) (1T = 795 800 A/m, μT = 0.7958 A/m), or more commonly in millitesla (mT) or microtesla

(μT) (WHO 2007). In some countries another unit called the gauss, (G), is commonly used (10,000 G = 1 T). The greater the current, the higher the strength of the magnetic flux density.

Extremely low frequency electromagnetic fields (ELF-EMF) exist when current flows (Hawkes et al. 2014). Typically, power lines and cables, residential wiring and electrical appliances produce these electromagnetic fields (Ahlbom et al. 2000). Most electric power operates at a frequency of 50 or 60 cycles per second, or hertz (Hz) (Redlarski et al. 2015). Close to certain appliances, the magnetic field values can be a few hundred μT (Havas 2002). Underneath power lines, magnetic fields can be about 20 μT. However, average residential magnetic fields in homes are much lower; about 0.07 μT in Europe and 0.11 μT in North America (WHO 2007).

In a residential setting, there are three major sources that produce ELF-EMF. These sources include the outdoor electrical distribution system consisting of either below or above ground wires and transformers, the indoor electrical distribution system, consisting of indoor wiring and grounding, as well as appliances (Havas 2004). Indoor electromagnetic exposure depends on the condition of the wiring and the specific room in a house. Electromagnetic exposure differs in rooms of a house with measurements ranging from 0.11 to 1.22 μT. A house with normal wiring does not usually exceed 0.03 μT (Riley 1995). Faulty wiring may lead to fields of 0.2 to 3 μT, with high readings near the walls, ceilings and floors of a house (Riley 1995; Havas 2002). The appliances of greatest concern are those with high magnetic flux densities and long exposure times. Electric blankets generate 2 to 4 μT and are in contact with the body for several hours each night. Hair dryers and electric shavers also generate high magnetic fields close the head (Havas 2004).

Occupational exposure to ELF-EMF varies amongst industries. The highest exposures have been measured in the textile, utility, transportation and metallurgical industries (WHO 2007). Dressmakers and tailors using industrial sewing machines are exposed to some of the highest magnetic flux

densities; in the order of 3 µT (IARC 2002). The magnetic flux densities encountered by utility workers; such as linemen, electricians, cable splicers, as well as power plant and substation operators, range from 1 to 4 µT, while metal workers, welders and aluminium galvanisers are exposed to values in the order of 2 µT (IARC, 2002). Railway workers are exposed to magnetic flux densities of up to 4 µT (IARC 2002). In the telecommunications industry, exposure is to relatively low magnetic flux densities ranging from 0.35 to 0.43 µT. Similarly, office workers, who come in contact with computers and photocopiers, are also exposed to relatively low magnetic flux densities of less than 0.17 µT (IARC 2002).

2.4 Biological effects related to extremely low frequency electromagnetic waves

Exposure to ELF-EMF has been implicated in various cellular and physiological effects in humans (Cui et al. 2014). All living cells comprise of molecules and atoms, which in turn comprise of electrons, neutrons and protons; giving cells their electrochemical nature (Genuis 2008). The homeostasis of cells, tissues and organs, rely on the ordered chemical and electrical activity of these molecules and atoms. Disturbances of these chemical and electrical activities in cells have the potential to disrupt cell functioning, metabolism and homeostasis by interfering with the normal physiology of required energy frequencies; leading to malfunction of organ systems, which may ultimately result in clinical illness (Genuis 2008).

Cellular pathogenesis as a result of ELF-EMF exposure is not completely understood (Genuis 2008). There still remains little agreement about the exact biological effects of ELF-EMF exposure and the mechanism underlying these effects (Santini et al. 2009). Various hypotheses have been proposed for the aetiology of cellular pathogenesis as result of ELF-EMF, based on preliminary evidence. Previously, it was believed that the underlying mechanism of the aetiology involves thermal

alteration of cells and tissue heating. However, increasing evidence suggests that ELF-EMF exposure could induce cell stress and cause damage on various intracellular components and mechanisms at the non-thermal level (Miyakoshi 2006; Calvente et al. 2010), although these suggestions of possible non-thermal effects remain controversial (Calvente et al. 2010).

Changes in protein synthesis following ELF-EMF exposure are similar to a cellular stress response (Blank 1995). The stress gene *HSP70* has been implicated in the cellular pathogenesis as result of ELF-EMF exposure. When cells are exposed to ELF-EMF, the stress gene *HSP70* is activated within minutes (Lin et al. 1997; Goodman and Blank 1998 Wei et al. 2016). The activation of this gene is accompanied by the binding of heat shock transcription factors to specific nucleotide sites in the promoter of the *HSP70* gene. The *HSP70* gene has two stimulus-specific domains that respond to two different physical stimuli. These stimuli are electromagnetic fields and increases in temperature (Lin et al. 1999). The DNA consensus sequences that respond to electromagnetic stimuli are nCTCTn (Lin et al. 1997; 1999). The existence of two different consensus sequences, pertaining to the two different stimulus-specific domains in the *HSP70* gene, is indicative of molecular evidence of different pathways that respond to non-thermal and thermal stimuli (Blank 2012).

Various authors have suggested a variety of biological responses to ELF-EMF. Some of these biological responses include the genotoxic effect of ELF-EMF that may influence DNA synthesis, DNA damage and RNA synthesis in many cell types (Conti et al. 1999; Mahrour et al. 2005; Genuis 2008; Shen et al. 2016) Other cellular responses to ELF-EMF include, alterations in cell proliferation and the cell cycle; the hindering of cell migration and adhesion, and alterations in the immune system (Blank 1995; Mahrour et al. 2005). Another interesting hypothesis is that ELF-EMF interferes with chemical reactions involving free radical production (Wolf et al. 2005). The pleiotropic effects of free radicals are well known and range from cytotoxic to mutagenic responses depending on the dose intensity, the duration of exposure, and the type of cell or tissue.

It is generally stated that energy exerted by ELF-EMF is too weak to interact with DNA and break chemical bonds in DNA molecules (McCann et al. 1997; Simkó et al. 2001; Cho and Chung 2003; Miyakoshi 2006; Zhang et al. 2016). The main cause of the scepticism is the low amount of energy that can be transferred by ELF-EMF (Dominici et al. 2011; Patruno et al. 2015). However, Santini et al. (2009) demonstrated that ELF-EMF do cause DNA damage (Santini et al. 2009), which is also supported by Zhu et al. (2001), who reported that ELF-EMF might induce DNA damage in lymphocytes. A dose-dependent increase in DNA breaks was demonstrated in animal brain cells with double strand DNA breaks appearing at 0.25 mT. The mechanism of interaction with DNA is probably through direct reaction with DNA (Rao et al. 2002). Specific mRNA level measurements in response to ELF-EMF exposure have shown to increase the levels of histone H3 and p53 mRNA (Cantini et al. 1986), IGF-II (Fitzsimmons et al. 1992), histone H2B, v-myc (Goodman et al. 1989), c-fos (Rao and Henderson 1996), and c-myc (Lin et al. 1996); supporting direct interaction with DNA (Rao et al. 2002).

The ELF-EMF potentially changes electrical charges of ions and molecules in cells, which may lead to the modification of ionic structures of elements within cell membranes, thereby disturbing the influx and efflux of various elements including calcium ions (Genuis 2008; Golbach et al. 2016). Thus, most theories suggest that because cell membranes play an essential role in mediating signal transduction events, they are by virtue of their bioelectrical properties the site where ELF-EMF exert their primary effects (Conti et al. 1999; Dini and Abbro 2005; Wolf et al. 2005). ELF-EMF are non-ionizing and are able to penetrate deeply into tissue (de Kleijn et al. 2011). It is thus plausible that ELF-EMF may affect membrane structure and permeability to small molecules (Wolf et al. 2005). It has been suggested that ELF-EMF influence ion- and membrane potential-dependent-processes, such as Ca^{2+} influx and efflux. These processes, which are responsible for the mediation of intracellular signalling (Conti et al. 1999; Wolf et al. 2005; de Kleijn et al. 2011), demonstrated an increase in Ca^{2+} concentration after ELF-EMF exposure. This increase in Ca^{2+} could be attributed to

over-expression of voltage-gated Ca^{2+} channels (VGCC). A number of studies have suggested that these VGCC maybe one of the direct targets of ELF-EMF (Pall 2013; Cui et al. 2014). Changes in VGCC may thus lead to changes in intracellular Ca^{2+} concentration (Morabito et al. 2010). An increase of intracellular Ca^{2+} concentration can lead to changes in the intra-cellular signalling mechanisms (Miyakoshi 2006; Pilla 2012; Pall 2013).

Based on the multitude of pleiotropic biological effects observed after exposure to ELF-EMF, Simkó and Mattsson (2004) presented a hypothesis suggesting an initial cellular event after ELF-EMF exposure. They suggested that ELF-EMF exposure results in a general cellular activation by means of increasing levels of free radicals. These authors further stated that exposure to ELF-EMF can cause both acute and chronic effects that are mediated by an increase in free radical levels. Direct activation by short-term exposure to ELF-EMF could lead to phagocytosis and macrophage activation; and consequently, free radical production. ELF-EMF could also increase the lifetime of free radicals resulting in persistently elevated free radical concentrations, thereby increasing the possibility of DNA damage. Long-term ELF-EMF exposure leads to a chronically increased level of free radicals, subsequently causing an inhibition of the effects of the pineal gland hormone melatonin. These induced reactions thus could lead to a higher incidence of DNA damage and therefore, to an increased risk of tumour development. In a related hypothesis by de Kleijn et al. (2016) proposed that ELF-EMF exposure can affect the hypothalamic pituitary-adrenal axis activation in mice as a result will affect number and activation of leukocytes.

2.5 Cancer related to extremely low frequency electromagnetic waves

Since the time that Wertheimer and Leeper (1979) published a report showing an increased risk of cancer mortality amongst children living near electrical wiring; many studies linking electromagnetic

fields to health effects have ensued. Although studies have linked ELF-EMF exposure with health effects, the debate is still continuing whether the levels of exposure in domestic settings could cause cancer, particularly childhood leukaemia (Angelillo and Villari 1999). Most epidemiologic studies have been concerned with childhood cancer, although linkages with other types of cancer have also been made.

Over the past three decades, potential health effects of residential and occupational exposure to ELF-EMF have been extensively investigated in epidemiological studies (Kheifets et al. 2010a). Infants and children are considered to be more vulnerable to environmental exposure of electromagnetic fields than adults (Calvente et al. 2010). Infant and childhood exposure have been implicated in numerous diseases raising concerns about the increased use of mobile phones and other household appliances (Zhao et al. 2014). Although most research has focused on a potential association between residential exposure and childhood leukaemia; an increased risk for childhood brain cancer has also been shown (Mezei et al. 2008).

2.5.1 Leukaemia

Ager et al. (1965) were the first authors who linked childhood leukaemia with environmental factors. However, Wertheimer and Leeper (1979) linked childhood cancer with electromagnetic fields. An excess of electrical wiring configurations, suggestive of high current-flow, was noted in Colorado in 1976 to1977 near the homes of children who developed cancer, as compared to the homes of control children. Wertheimer and Leeper (1979) found that children exposed to ELF-EMF had a 2-fold risk of developing leukaemia, as opposed to children with lower exposures. Their findings were strongest for children who had spent their entire lives at the same address, and it appeared to be dose-related.

Leukaemia is the most common malignancy diagnosed in children under the age of 15 years (Ahlbom et al. 2000). Leukaemia is divided into acute and chronic types, and acute lymphocytic leukaemia (ALL). The majority of childhood leukaemia cases (80%) are of the ALL type; primarily in children of ages 1–4 years (Metayer et al. 2013). ALL is characterised by the presence of immature blast cells in the bone marrow, blood and other tissues (Zelig et al. 2011). ALL is therefore a disorder of the lymphopoietic stem and progenitor cells originating from the bone marrow, thymus and lymph nodes (Pyatt and Hays 2010).

The hypothesised risk factors associated with ALL include infections, benzene exposure, vehicle exhaust emissions, allergies, non-ionizing radiation and exposure to electromagnetic fields (Pyatt and Hays 2010). It has been shown that only 5% of ALL aetiology can be attributed to genetic disorders, chemotherapeutic agents and ionizing radiation (Pyatt and Hays 2010). It has also been suggested that the initial etiological event occurs in utero (Ziegelberger et al. 2011). Ding and Bao (2013) proposed that gene-environmental interactions may play a role in the aetiology of this disease. Globally, the incidence of ALL is increasing, also exhibiting geographic and ethnic differences (Pyatt and Hays 2010). Increased incidences are favoured in the industrialised countries over developing countries (Calvente et al. 2010). For example, the occurrence of ALL in the developing country Nigeria is low, as opposed to Denmark, a developed country, showing a 30-fold increase (Pyatt and Hays 2010).

Various meta-analyses have indicated an epidemiological association between residential exposure to magnetic fields and the risk of childhood leukaemia (Kheifets et al. 2010a; Schüz and Erdmann 2016). Particularly, the overall risk of leukaemia increased in children exposed to magnetic fields > 0.3 μT (Greenland and Sheppard 2000) and ≥ 0.4 μT (Zhao et al. 2014). Studies have also shown that the risk for childhood leukaemia increases in the proximity of electromagnetic power sources. For example, in England and Wales the relative risk (RR) was 1.68 at 200 m from high-voltage

power lines, while the RR reduced to 1.22 at a distance of 200–600 m (Draper et al. 2005). These results were supported by a study in northwest Iran where the risk increased for childhood leukaemia in areas < 500 m from a high-voltage power lines (Feizi and Arabi 2007).

The risk for childhood leukaemia increases in children born from mothers exposed to electromagnetic fields during pregnancy. It was shown in a study between 1980 and 1993 in Quebec, Canada, that the risk of a child developing leukaemia increases when a pregnant mother was exposed to levels of electromagnetic fields ≥ 0.4 μT (Infante-Rivard and Deadman 2003). In another study, Feychting et al. (2000) showed that parents exposed to electromagnetic fields with levels ≥ 3 μT, the risk for leukaemia developing in their children increased, although the risk only reached significance in boys.

The association between exposure to ELF-EMF and childhood leukaemia has led to the classification of magnetic fields by the International Agency for Research on Cancer (IARC) as a "possible human carcinogen" (Kheifets et al. 2006). This association is regarded as the critical effect in risk assessment.

A number of large studies have also linked adult leukaemia with exposure to ELF-EMF. In a study of 2.8 million unselected Danes the occupational exposure to ELF-EMF was followed up over a 17 year period; from 1970 to 1987 (Guenel et al. 1993). Each person was classified according to his or her industrial occupation as either being intermittently exposed or continuously exposed to ELF-EMF. An increased risk of leukaemia in some occupations working with electricity could be demonstrated. These occupations included electricians, welders and flame cutters, aluminium workers, power and telephone linemen, electrical engineers, electronic technicians, radio and telegraph operators, and power station operators. In a small case-control study in New Zealand, an almost 3-fold increase in the risk for leukaemia was shown amongst welders and flame cutters (Bethwaite et al. 2001;

Johansen et al. 2007). In a Dutch cancer incidence study 120,852 men and women aged 55–69 years at time of enrolment in 1986, were followed up 17.3 years later (Koeman et al. 2014). An increased risk was indicated for acute myeloid leukaemia amongst men with occupational ELF-EMF exposure.

2.5.2 Breast cancer

In the developed and developing world, breast cancer has been ranked worldwide as the fifth cause of death amongst all cancers (Girgert et al. 2005; Ferlay et al. 2010). Since the middle of the last century, the incidence of cancer has risen by 1% in the United States of America (Caplan et al. 2000). This rise cannot to be explained completely by the known risk factors. This has led to numerous studies investigating the possible contribution of exposure to ELF-EMF produced by electric power facilities and household appliances (Caplan et al. 2000). In the past two decades, many epidemiological studies have shown the existence of a relationship with an increased risk of adult breast cancer and exposure to ELF-EMF, although these contentions remain controversial (Ottini et al. 2010). In animal studies, animals with breast cancer have demonstrated more tumours and larger tumours when exposed to ELF-EMF (Ottini et al. 2010).

Breast cancer is the most frequent non skin cancer in women worldwide (Althuis et al. 2005; Porter 2009). Studies have shown that premenopausal woman exposed to residential ELF-EMF and ELF-EMF emitted from power lines may have an increased risk of breast cancer (Kliukiene et al. 2004; Girgert et al. 2005; Chen et al. 2013). In two studies, premenopausal women exposed to environmental fields stronger than 0.2 μT, showed an increased risk of breast cancer (Wertheimer and Leeper 1982; Feychting and Ahlbom 1993). In device measurement-based studies, a slight increased risk was found only in premenopausal breast cancer (Zhang et al. 2016). However, the converse was shown in studies conducted in Finland and Taiwan, where no link could be

established between populations living approximately 100 to 500 m from power lines and any increased risk of breast cancer in woman (Verkasalo et al. 1996; Li et al. 1997).

Melatonin is a central component in circadian rhythms and helps control the sleep and wake cycles of a person. It is a hormone secreted by the pineal gland in the brain. It has been reported that a reduced expression of melatonin could be involved in carcinogenesis and reproduction problems (Reiter 1998; Levallois et al. 2001; Manzetti and Johansson 2012). A study during 2001 showed that melatonin levels in women living near power lines were lower than melatonin levels in women living away from any power lines (Erren 2001). It has been hypothesised that ELF-EMF may increase the risk of breast cancer by reducing the production of melatonin (Stevens and Davis 1996; Forssén et al. 2005; Li et al. 2013; Proietti et al. 2013). Radio and television broadcasting transmitters have been shown to reduce melatonin metabolites in women living in close proximity of these ELF-EMF transmitters (Manzetti and Johansson 2012). For a decade, there has been evidence that human breast cancer cells grow faster if exposed to ELF-EMF, probably because of the reduced melatonin levels in the body (Hardell and Sage 2008).

With the increased use of electric devises in the household, such as electric bed-warming blankets, many studies have been conducted to ascertain if a link existed between the ELF-EMF of electric bed-warming blankets and breast cancer in woman. Most of these studies could not find an association with the risk for breast cancer (Vena et al. 1991; 1994; Gammon et al. 1998; Laden et al. 2000; Davis et al. 2002; Chen et al. 2013; Feychting 2013). Similar results were obtained for pre- and postmenopausal women and for women with oestrogen-receptor positive breast cancer (Laden et al. 2000; Forssén et al. 2005).

Breast cancer in men is rare; however the incidence of male breast cancer has risen over the past few decades (Ottini et al. 2010; Sun et al. 2013). Epidemiologic studies have demonstrated an

association between ELF-EMF exposure and male breast cancer (Ottini et al. 2010; Sun et al. 2013). In contrast, some studies found no or a weak association between ELF-EMF and male breast cancer (Johansen and Olsen 1998; Floderus et al. 1999; Pollán et al. 2001; Nichols and Sorahan 2005). The major risk factor for male breast cancer is a positive family history, because currently little is known about other risk factors such as those involving hormones and the environment (Ottini et al. 2010).

2.5.3 Brain cancer

The highest rates of brain cancer have been noted in the more developed areas of the globe. In Europe, Australia/New Zealand, and North America, the incidence of new cases each year is around 7.5–14 cases per 100,000 of the population's males and 4–11 new cases for females. In contrast, in the developing world, for example Africa and the Pacific islands, the incidence of brain cancer is the lowest (Genuis 2008; Filippini 2012).

The association between brain cancer and ELF-EMF from residential and environmental emissions has been investigated in a number of studies. A meta-analysis of 48 studies published during 1993 to 2007 reported a small positive association between occupational ELF-EMF and brain tumours (Kheifets et al. 2008; Turner et al. 2014). Contradicting outcomes have also been reported, where little or no association between ELF-EMF and brain cancer in adults could be established (Kheifets 2001; Kheifets et al. 2010b). However, a meta-analysis of occupational studies indicated a slightly higher risk for electrical workers (Genuis 2008). Astrocytoma and meningioma were the most common types of brain cancer associated with ELF-EMF exposure (Thériault et al. 1994; Floderus et al. 1999; Havas 2004; Baldi et al. 2011). Studies of residential exposure to ELF-EMF and childhood brain tumours have also produced inconsistent results (Filippini 2012). Most recent studies provide little evidence of an association between childhood brain cancer and ELF-EMF (Kheifets 2001; Kheifets et al. 2010b).

2.6 Other health-related effects related to extremely low frequency electromagnetic waves

2.6.1 Haematology and immunology

All blood cells are derived from haematopoietic stem cells in the red bone marrow. Haemopoiesis is the process in which all blood cells are formed through progressive division and maturation of haematopoietic stem cells. Three blood cell lineages exist, namely, erythroid cells, lymphoid cells and myeloid cells (Marieb and Hoehn 2016). Erythroid cells are the oxygen carrying red blood cells, while the lymphoid cells are composed of T-lymphocytes and B-lymphocytes, which form the foundation of the immune system (VanPutte et al. 2016). Lymphocytes express different protein receptors on their cell surfaces (antigens), which are referred to as clusters of differentiation (CD). These CD antigens participate in immune reactions as receptors for cellular communication. Myeloid cells include the granulocytes, megakaryocytes and macrophages and are involved in innate immunity, adaptive immunity and blood clotting. The nonspecific mechanism that is activated immediately or within hours of an antigen's appearance in the body is referred to innate immunity; while the adaptive immune system, also known as the acquired immune system, is a highly specialised mechanism that eliminates or prevents pathogen growth (Marieb and Hoehn 2016).

When men were exposed to ELF-EMF, blood profiles did not reveal significant differences when compared to control groups. An examination of the profiles of haemoglobin concentration, haematocrit, red blood cells, platelets, total leucocytes, monocytes, lymphocytes, eosinophils and neutrophils suggested that these magnetic fields do not have a cumulative effect on these blood factors (Selmaoui et al. 1996). Touitou et al. (2013) found similar results for profiles of red blood cells, haemoglobin concentration, haematocrit, platelets, mean platelet volume, total white blood cells, lymphocytes, monocytes, eosinophils, basophils and neutrophils. A study carried out on 16

male welders also could not establish significant effects of ELF-EMF on red blood cells, haemoglobin, haematocrit, platelets, total white blood, neutrophils, lymphocytes and eosinophils (Dasdag et al. 2002).

In contrast to the human studies, some significant differences could be established for blood factors in mice. Exposure of mice bone marrow cells to ELF-EMF showed a reduction in the proliferation and differentiation of the granulocyte-macrophage progenitors when compared to non-exposed bone marrow cells (van den Heuvel et al. 2001). In another study, a significant decrease in the counts of monocytes, platelets, peripheral lymphocytes, as well as splenic total T- and B-lymphocytes levels were observed (Hashish et al. 2008).

Various studies have been conducted to investigate the influence of ELF-EMF on immunological parameters. The vast majority of these studies could not identify an association between these magnetic fields and the immune system. Selmaoui et al. (1996) found no effects on the immunological parameters CD3, CD4, CD8, natural killer cells and B-lymphocytes in 16 healthy men aged 20–30 years. Similar results were found for 15 men exposed to ELF-EMF (Touitou et al. 2013). No cumulative effects could be established for CD3, CD4, CD8, natural killer cells, B-lymphocytes, total CD28, $CD8^+$, $CD28^+$ and activated T-lymphocytes. In a study carried out on 16 male welders, the results also suggested that ELF-EMF do not affect the immunologic parameters CD3, CD4, CD8 and CD4/CD8 (Dasdag et al. 2002). Contrary to these studies, a few studies have suggested some effects on the immune system. When Bonhomme-Faivre et al. (1998a) monitored ELF-EMF exposure in hospital laboratory workers, they observed lowered CD3 and CD4 lymphocyte counts, but increased natural killer cell counts. The numbers of natural killer cells and oxidative bursts of monocytes, which are implicated in cytotoxic responses, were significantly increased in 10 men working with induction heaters, while monocytes had significantly reduced phagocytic activity compared with those from unexposed workers (Tuschl et al. 2000). These authors concluded that

overall the non-specific immunity of the exposed workers was normal and that the most peculiar finding was the increase in natural killer cells. A study of 60 workers of power utilities revealed that there was an association between exposure intensity and a decreased ornithine decarboxylase activity and lowered natural killer cell counts (Ichinose et al. 2004).

It has been shown that ELF-EMF have an effect on components of the immune system *in vivo* by inducing changes in blood cell levels in both mice and rats. This effect was probably caused by lymphocyte proliferation (Hashish et al. 2008). It has also been suggested that ELF-EMF might influence macrophage functioning *in vitro* by increasing free radical production and stimulating phagocytic activity (Frahm et al. 2006; 2010). Thus, it has been concluded that ELF-EMF exposure may modulate the innate immune response to microorganisms in animal models (de Kleijn et al. 2011).

2.6.2 Endocrine system

Amongst the many hormones secreted by the human body, the neuroendocrine hormone melatonin has been the focus of most of the ELF-EMF exposure studies. Melatonin is a tryptophan derivative produced mainly by the pineal gland near the third ventricle in the brain. Melatonin is involved with regulation of multiple physiological processes, which include sleep patterns, free radical metabolism, blood pressure control, nitric oxide physiology, lipid metabolism, immune system functioning and the activity of sex hormones (Miyakoshi 2006). The melatonin secretion is generally regarded as being particularly sensitive to electric, magnetic and electromagnetic field exposure (Lewczuk et al. 2014).

Several studies have shown that ELF-EMF exposure results in the reduction of melatonin production. A study into the effect of ELF-EMF on melatonin production in new-borns kept in incubators for at least 48 hours revealed that the melatonin levels increased almost immediately after the babies were taken out from the incubators (Bellieni et al. 2012). This result highlights the

potential for ELF-EMF exposure to influence the production of melatonin. Melatonin levels dropped significantly in repairers of electronic equipment exposed to ELF-EMF when compared to the controls (El-Helaly and Abu-Hashem 2010; Singh and Kapoor 2014). A reduction in the urinary melatonin metabolite, 6-sulfatoxymelatonin, was demonstrated in electrical utility workers (Burch et al. 1998; 1999; 2000; Lewczuk et al. 2014). This change in melatonin metabolite concentrations was noted after the second day of the working week and the effect of the magnetic field exposure was the most prominent in subjects with low workplace light exposures. One hypothesis that has been put forward to explain the inhibitory effect of electromagnetic fields on melatonin production is that the radical scavenging effect of melatonin is attenuated. However, at present, this hypothesis has not been definitively confirmed (Miyakoshi 2006).

It has been hypothesized that the risk of breast cancer increases when melatonin production is reduced (Li et al. 2013). The alteration of melatonin production could change the risk of breast cancer through its regulation of reproductive hormones by influencing the hypothalamic-pituitary-gonadal axis. Decreased concentrations of circulating melatonin may result in increased secretions of gonadotrophins by the pituitary gland and increased levels of ovarian hormone production by the ovaries, which could ultimately increase the risk of breast cancer (Tamarkin et al. 1981).

2.6.3 Nervous system

Evidence has suggested that 4.6 million new neurodegenerative disease cases appear globally every year (Ferri et al. 2005). Alzheimer's disease is regarded as the most common of the dementias, contributing to about 75% of all cases (Mattsson and Simkó 2012). Neurodegeneration is a progressive loss of structure and function of neurons (Cannon and Greenamyre 2011; Mattsson and Simkó 2012). Although genetic factors play a role in the development of various neurodegenerative diseases, evidence suggest that occupational and environmental factors also contribute to the development of these diseases (Johansen and Olsen 1998; Davanipour et al. 2007;

Cannon and Greenamyre 2011; Mattsson and Simkó 2012). Neurodegenerative diseases that have been associated with ELF-EMF exposure include Alzheimer disease, Parkinson disease and amyotrophic lateral sclerosis (Cannon and Greenamyre 2011; Yin et al. 2016). These diseases all involve the death of specific neurons, although their aetiology seems to be different (Savitz et al. 1998a; 1998b; Say et al. 2016).

Alzheimer disease is a progressive degenerative disorder resulting from the degeneration of neurons causing loss of memory and cognitive abilities, as well as behavioural changes (WHO 2007). Studies investigating a possible association between Alzheimer disease and ELF-EMF are limited. In a study conducted with 1,527 patients at the Californian Alzheimer's Disease Diagnostic and Treatment Centres, Davanipour et al. (2007) demonstrated an increased risk to Alzheimer disease resulting from elevated occupational exposure to ELF-EMF. In a systematic review and meta-analysis of 14 different studies, evidence suggested an association between occupational exposure to ELF-EMF and Alzheimer disease (García et al. 2008).

Parkinson disease is a degenerative disorder of the central nervous system mainly affecting the motor system and is characterised by motor dysfunction, tremor, muscular rigidity and slow imprecise movements (WHO 2007). Little is known about the influence of ELF-EMF and Parkinson disease. However, Noonan et al. (2002) reported that an association between occupational ELF-EMF exposure and Parkinson's disease after studying males in the state of Colorado for the years 1987 to 1996.

Researchers have since the late 1970s been interested in the chronic health effects of ELF-EMF on psychiatric disorders. Many contradicting results have been published about the incidence of suicide and depression and their association with ELF-EMF exposure (van Wijngaarden et al. 2000). However, a few studies did reveal a strong association between ELF-EMF and suicide and

depression. Van Wijngaarden et al. (2000) provided evidence for a strong association between occupational electromagnetic fields and suicide amongst male electric utility workers, while Dowson et al. (1988) found a strong association between depression amongst people who lived near power lines in England.

2.6.4 Skeletal and muscular effects

Studies have demonstrated a therapeutic effect on bone repair by applying ELF-EMF. Bassett and his colleagues (1965;1967;1974) were one of the first groups to show that bone repair was augmented by electromagnetic fields. Bassett et al. (1974) showed that bone formation took place in areas of negative charge, while bone remodelling occurred in zones of positive charge. Although a full understanding of the underlying mechanisms behind these beneficial effects of ELF-EMF on bone repair is unclear, some important breakthroughs have been made (Santini et al. 2009). Preclinical studies have shown that electric and electromagnetic fields regulate proteoglycan and collagen synthesis in models of endochondral ossification, and increase bone formation *in vivo* and *in vitro* (Aaron et al. 1989; 2004). ELF-EMF accelerate bone formation and healing, particularly osteotomies and spine fusions. *In vitro* studies have shown the pulsed EMF stimulate osteoprogenitor cells and osteoblasts to proliferate and differentiate (Dimitriou and Babis 2007; Pall 2013).

To investigate the effect of ELF-EMF on muscle cells, single cells were exposed to ELF-EMF. C2C12 cells were used as an *in vitro* model of the skeletal muscle phenotype (Morabito et al. 2010). Exposure of these cells to ELF-EMF revealed an induced reactive oxygen species production in myoblasts and myotubes. A concomitant decrease in mitochondrial membrane potential was demonstrated, which activated the cellular detoxification system, thereby increasing catalase and glutathione peroxidise activities. This resulted in the alteration of intracellular Ca^{2+} homeostasis, by increasing spontaneous activity of myotubes and enhancing cellular reactivity.

A study of the effects of ELF-EMF on rat myocardial cells revealed changes in the myocardium. ELF-EMF caused oxidative stress, apoptosis and morphologic damage in myocardium of adult rats (Kiray et al. 2013). These changes in the myocardium could be attributed to increased oxidative stress.

2.6.5 Cardiovascular system

Although most epidemiologic studies have shown no effect of ELF-EMF on cardiovascular changes (Kheifets et al. 2007; McNamee et al. 2009), a large study conducted by Savitz et al. (1999) revealed an opposing outcome. The mortality from cardiovascular disease in relation to occupational magnetic field exposure amongst a cohort of 138,903 male electric utility workers from five USA companies over the period 1950 to 1988 was examined. The data from this study suggested a possible association between occupational magnetic fields and arrhythmia-related heart disease.

2.6.6 Reproduction and new-borns

Foetal development depends on an array of complex physiological processes and interactions. An adverse outcome of a pregnancy results because of complex interactions between maternal, placental, foetal and environmental factors present during pregnancy (de Boo and Harding 2006). These interactions are poorly understood. It is known that endogenous electric fields play a role in normal embryo development, by for example, guiding cell orientation and migration. Developmental abnormalities may thus result from disruptions from these electric fields (Saunders and McCaig 2005). Whether ELF-EMF exposure during pregnancy could have a similar effect is still unclear (Feychting 2005). Although uncertainty of the causal agent exists, an increased risk of miscarriage has been linked to environmental ELF-EMF exposure (Li et al. 2002; Auger et al. 2011; 2012). In a study amongst pregnant women within a large health maintenance organisation in San Francisco, an

increased risk for miscarriage was shown with increasing levels of maximum magnetic field exposure with a threshold around 16 mG (Li et al. 2002).

ELF-EMF exposure during pregnancy can affect new-borns adversely. Residential exposure to ELF-EMF from high voltage cables, overhead power lines, electricity substations or towers have been associated with reduced birth weight and may be associated with adverse birth outcomes or even miscarriages (de Vocht and Lee 2014). A study of 140,356 singleton live births in Northwest England from 2004 to 2008 suggested that living close to residential ELF-EMF sources reduced average birth weight, but not with statistically significant increased risks for other adverse perinatal outcomes (de Vocht and Lee 2014). New-borns exposed to incubator conditions also demonstrated increased heart rate variability (Bellieni et al. 2008).

Exposure to ELF-EMF has also been linked to testicular development. A study amongst switchyard workers revealed an increase in the number of chromosomal aberrations and an increased tendency towards malformations amongst their children. Fathers employed in industries with a higher exposure to ELF-EMF than average also tend to have children with higher rates of brain and spinal cord tumours (Genuis 2008). In rats, exposure to ELF-EMF cause significant histopathological alterations, such as focal tubular atrophy, necrosis, and seminiferous epithelial erosion (Erpek et al. 2007). In male rat offspring, a reduction in the count, diameter, area and volume of seminiferous tubules, and height of seminiferous epithelium along with Leydig cell count, was indicative of the harmful effects of ELF-EMF on testis development (Tenorio et al. 2011).

2.7 Epidemiological studies

All epidemiologic studies to date have been based on retrospective assessments of exposure to ELF-EMF. It is unlikely that researchers will ever perform prospective research; given the potential health outcomes of such studies (Ahlbom et al. 2001). A large number of retrospective epidemiologic

studies have been conducted over the years to ascertain if associations existed between exposure to ELF-EMF and specific health effects, such as cancer, cardiovascular diseases and neurodegenerative diseases. Many of these studies are meta-analyses combining several previous studies. Table 2.2 provides a summary of several large epidemiological studies investigating the possible association between ELF-EMF exposure and health effects. Because many epidemiologic studies have indicated that ELF-EMF affects human health in one way or another; studies to elucidate the effects of ELF-EMF at a molecular and cellular level will continue. In conclusion, because of the lack of experimental evidence of biological studies and the contradictory reported results of epidemiological studies, this research project was undertaken to elucidate the effect of ELF-EMF at the cellular biological level.

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Table 2.2 Summary of epidemiological studies.

Exposure effect	Period of study / total number meta-analyses	Study population	Outcome	Reference
Cancer risk	42 studies	13,259 cases 100,882 controls	Exposure to ELF-EMF increases the risk for cancer, particularly for premenopausal breast cancer.	Zhang et al. (2016)
Childhood leukaemia	9 Studies	3,203 cases 10,338 controls	Exposure to ≥ 0.4 μT ELF-EMF increases the risk for childhood leukaemia.	Ahlbom et al. (2000)
	1997–2013 9 studies	11,699 cases 13,194 controls	Positive association between exposure to ≥ 0.2 μT ELF-EMF and childhood leukaemia.	Zhao et al. (2014)
	1988–1996	1,842 cases 3,099 controls	Hypothesis that night-time increases risk for childhood leukaemia could not be supported.	Schüz et al. (2007)
	1962–995	29,081 cases 9,700 controls	Exposure to ELF-EMF increases the risk for childhood leukaemia for children living within 200 m of power lines.	Draper et al. (2005)
Adult leukaemia	1982–1998 22 studies	Not divulged	Association between field exposure to ELF-EMF and adult leukaemia was inconclusive.	Wang et al. (2000)
Breast cancer in women	1990–2010 23 studies	74,975 cases 249,281 controls	Exposure to ELF-EMF increases the risk for female breast cancer, especially for premenopausal and oestrogen receptor positive females.	Chen et al. (2013)
	1966–2000 24 studies	> 5 M cases > 5 M controls	Exposure to ELF-EMF increases the risk for female breast cancer.	Erren (2001)

Exposure effect	Period of study/total number meta-analyses	Study population	Outcome	Reference
Breast cancer in men	1966– 2000 15 studies	> 5 M cases > 5 M controls	Exposure to ELF-EMF increases the risk for male breast cancer.	Erren (2001)
	1991–2012 18 studies	7 case-control studies 57 cases 223 controls 11 cohort studies 299 cases total of 7,486,643	Exposure to ELF-EMF increases the risk for male breast cancer.	Sun et al. (2013)
	1970–1987	2.8 M cases	Exposure to ELF-EMF increases the risk for male breast cancer.	Guenel et al. (1993)
Depression and suicide	1950–1986	138,905 cases 5,348 controls	Positive association between occupational exposure to ELF-EMF and suicide.	van Wijngaarden et al. (2000)
Brain cancer	2000–2004	3,761 cases 5,404 controls	Positive association between exposure to ELF-EMF and glioma.	Turner et al. (2014)
Cardio-vascular effect	1950–1988	138,903 cases	Positive association between exposure to ELF-EMF and arrhythmia-related heart disease.	Savitz et al. (1999)
Reproductive effect	2004– 2008	140,356 singleton live births	Positive association between close residential (≤50 m) exposure to ELF-EMF and reduced birth.	De Vocht and Lee (2014)

Chapter 3

Materials and Methodology

3.1 Introduction

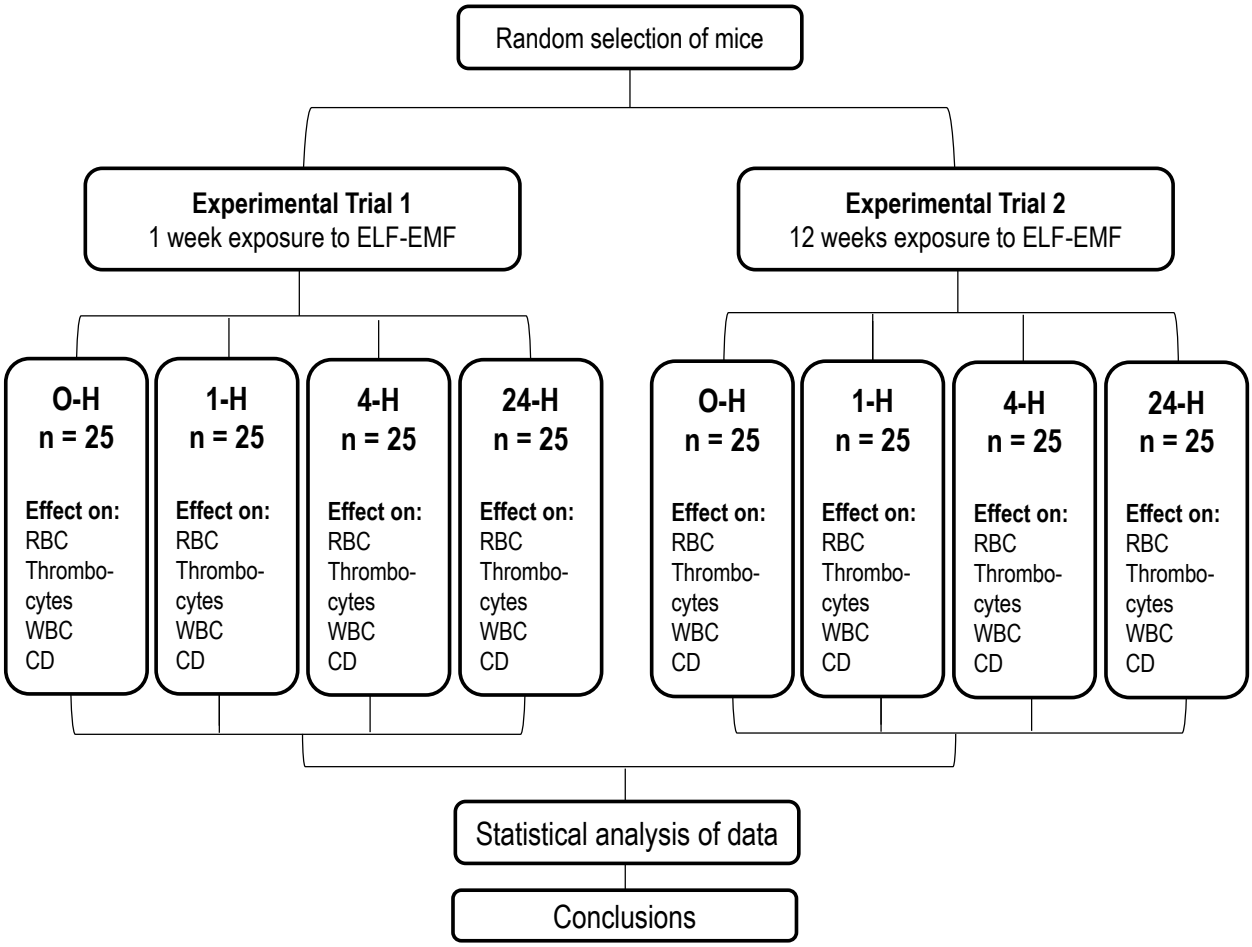
Exposure to ELF-EMF has been implicated in various cellular and physiological effects in humans (Cui et al. 2014). However, cellular pathogenesis as a result of ELF-EMF exposure is not completely understood (Genuis 2008). Thus, this project was undertaken to study the effects of ELF-EMF on a number of cellular parameters in mice (*Mus musculus*). The cellular parameters included the number of erythrocytes (red blood cells; RBC); number of thrombocytes (platelets; PLT) and the number of the different classes of leucocytes (white blood cells; WBC). Four immunophenotyping cluster of differentiation (CD) classes, as well as the haemoglobin concentration, haematocrit and mean corpuscular volume were also included.

This study forms part of a larger study, which focused on the biological effects of extremely low frequency electromagnetic field (ELF-EMF) exposure in mice. This research project was conducted under the auspices of three collaborating parties; the Central University of Technology, Free State, from South Africa; the University of Wageningen from the Netherlands and the Engineering Company Immunent BV, also from the Netherlands.

3.2 Experimental design

In this project, two experimental trials were performed. In Experimental Trial 1, mice were exposed to ELF-EMF for one week, while in Experimental Trial 2; mice were exposed for a 12-week period (Figure 3.1). The magnetic flux density applied in both experimental trials was 5 μT. In each experimental trial, four ELF-EMF exposure treatment regimens were applied to groups of 25 randomly selected mice. These treatment regimens were as follows:

- A non-exposure treatment (control treatment);
- A daily 1-hour exposure treatment from 8:00 until 9:00;
- A daily 4-hour exposure treatment from 8:00 until 12:00; and
- A 24-hour continuous exposure treatment.



0-H = 0-hour exposure; 1-H = 1-hour exposure; 4-H = 4-hour exposure; 24-H = 24-hour exposure

Figure 3.1 Study design.

3.3 Experimental animals and treatment facilities

3.3.1 Experimental animals

The experimental mice used in this study were supplied by the Animal Unit of the School of Medicine in the Faculty of Health Sciences, at the University of the Free State, Bloemfontein, South Africa. The mice used in both experimental trials belonged to the BALB/c N_1H strain. This mice strain is an inbred albino strain, specifically bred for the testing of a variety of blood parameters. Only 4-week old healthy males were exposed to the various ELF-EMF treatment regimens.

3.3.2 Treatment rooms

Both experimental trials were conducted at the Animal Unit of the School of Medicine in the Faculty of Health Sciences, at the University of the Free State. The experimental trials were conducted in two treatment rooms. These treatment rooms were isolated from all other activities in the Animal Unit. The one treatment room was used as the control treatment room; where mice were not exposed to ELF-EMF. This treatment regimen was referred to as the 0-hour control treatment or control. In the other treatment room all three ELF-EMF treatment regimens were applied to the mice; 1-hour, 4-hour and 24-hour exposures. The two treatment rooms were positioned approximately 20 metres apart to ensure that the ELF-EMF in the room in which mice were exposed to ELF-EMF did not reach the room containing the control treatment group of mice. Furthermore, to avoid interference with the ELF-EMF, no other metal components were used in the construction of either of the two treatment rooms.

The environmental conditions of the respective treatment rooms are centrally controlled in the Animal Unit. For these experiments, a 12-hour day-night cycle was implemented. The two treatment rooms were also kept at a temperature ranging from 18 to 22°C and a humidity of 50 ± 10%. Furthermore, each treatment room was supplied with a constant flow of filtered air. A concerted

effort was made to keep noise to a minimum so that the stress levels of the animals were as kept as low as possible.

Access to the treatment rooms was kept to a minimum and restricted to researchers participating in the project. Researchers wore protective clothing; masks and gloves, when entering treatment rooms. At the entrance of each treatment room, a sterilising footbath was placed into which a researcher stepped before entering a treatment room.

3.3.3 Animal exposure cylinders

For the exposure of mice to ELF-EMF, specific housing cylinders were designed. Each housing cylinder could contain three mice cages. These housing cylinders were constructed from polyvinyl chloride (PVC) and were approximately 120 cm long with a diameter of 30 cm. Each cylinder was perforated along its length with evenly spaced 2 cm diameter holes to provide light and air to the mice. To supply the ELF-EMF, 30 coils of copper wire were coiled around each of the PVC cylinders (Figure 3.2).

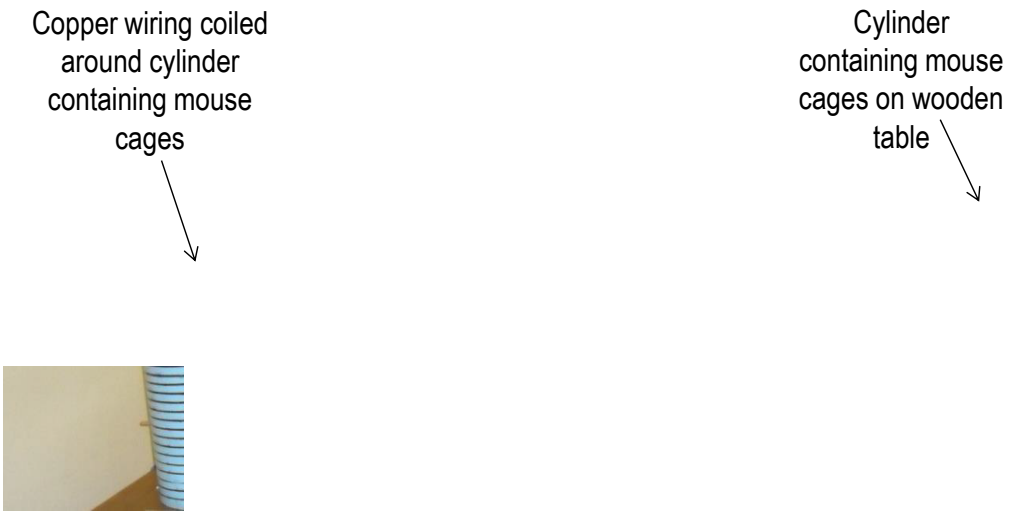


Figure 3.2 **Treatment room showing blue PVC cylinders containing animal cages.** The copper wiring coils are clearly visible around a PVC cylinder.

Three specifically designed mice cages were stacked on top of one another in each PVC cylinder. Each of the mice cages was placed on wooden rods that were threaded through holes in the PVC cylinder. These mice cage containing PVC cylinders were then placed on wooden tables in the two treatment rooms. The control treatment room contained four PVC cylinders, while the ELF-EMF exposure room contained 12 PVC cylinders.

The 12 PVC cylinders in the ELF-EMF exposure room were connected to an electromagnetic field generator. This electromagnetic field generator was supplied by Immunent Signal System, Chamber of Commerce register number 17137077 and patent number WO 03/035176. The electrical signal from the Immunent Signal System was delivered to the copper coils surrounding each of the PVC cylinders providing electromagnetic field homogeneity of < 0.4% over the mice cages (Figure 3.3). At the centre of a particular PVC cylinder coil, the mean magnetic flux density was 5 μT. The magnetic flux density was checked daily by an engineer with a F.W. Bell Gauss/Tesla meter probe. Outside the PVC cylinders, the magnetic flux density diminished rapidly and did not overlap with adjacent PVC cylinders. Heat or sound development was negligible at this low magnetic flux density.

Figure 3.3 Immunent signal system with PVC cylinder with copper coils.

3.3.4 Animal cages

The mice were housed in polypropylene mouse cages. These cages were specially designed to fit inside a PVC cylinder. A mouse cage was approximately 23 cm long, 15 cm wide and 12.5 cm high (Figure 3.4). To ensure that animals were not shocked during exposure, all parts of a mouse cage was non-metallic. The Perspex mouse cage lid comprised of two small troughs; one of the troughs contained the feeding pellets and the other for the water bottle.

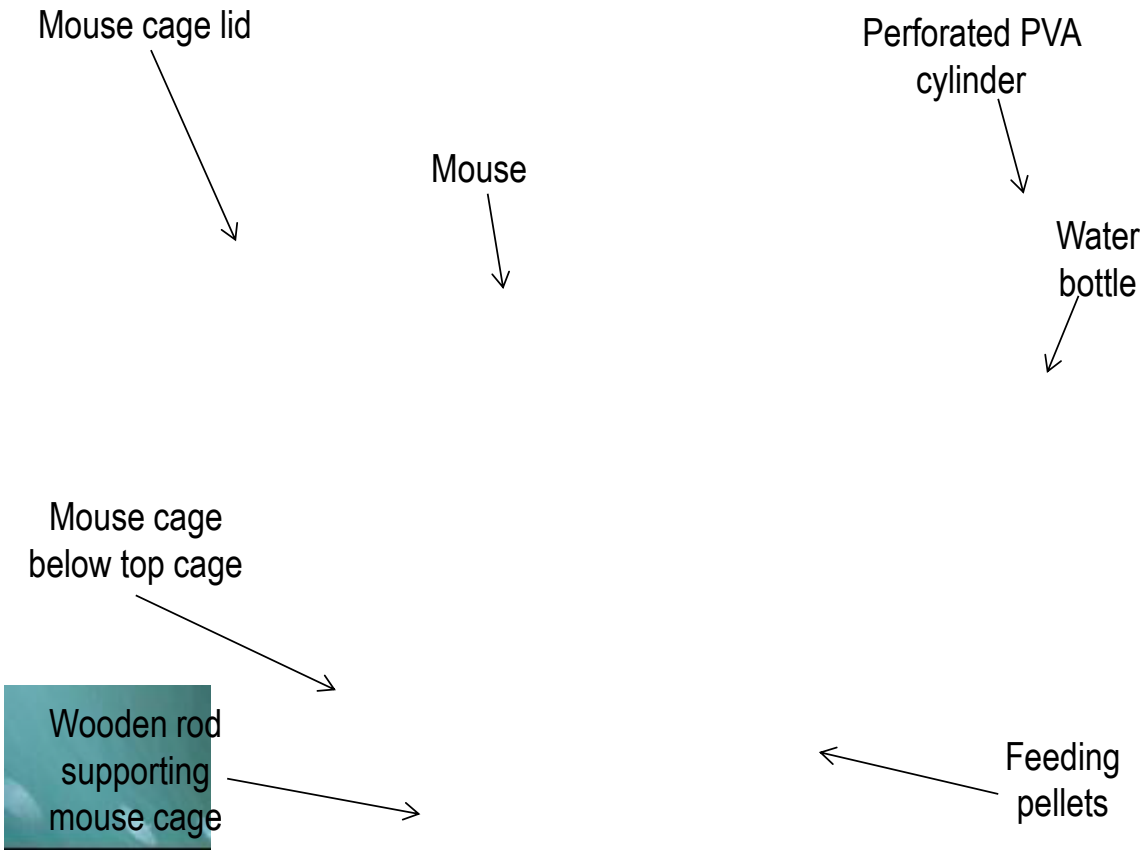


Figure 3.4 Specially designed polypropylene mouse cage.

3.4 Methods

3.4.1 Set up of experimental trials

For this project, mice were randomly selected for each of the two experimental trials from four-week-old male mice. Before the random selection of mice commenced; a weight range was determined which was used to guide the selection of mice. This weight range was determined by weighing four hundred four week-old-male mice, and thereafter calculating the mean weight and standard deviation. For both experimental trials, only mice weighing within the range of the mean weight of the 400 mice $\pm$ two standard errors were chosen. Thus, the weights of all mice used in this study ranged from 26.03 to 27.99 g ($\bar{X} \pm 2SE$; 27.01 $\pm$ 0.98).

Both experimental trials comprised of four treatments. One treatment was considered to be the control treatment, in which the mice were not exposed to ELF-EMF (0-hour control treatment). The other treatments comprised of exposure to ELF-EMF for one hour (1-hour treatment), four hours (4-hour treatment) and continuous exposure for 24 hours (24-hour treatment). For each treatment, 25 mice were randomly selected. Mice falling within the weight range were assigned to each of the four treatments one-by-one in rotation for a particular experimental trial. Once mice had been assigned to the respective treatments, an analysis of variance was performed to ascertain if the mean weights of the different treatment groups within each experimental trial were not significantly different. The ANOVA tests revealed that there were no significant differences ($\boldsymbol{p}$ > 0.05) between the mean weights of the treatment groups within each of the two experimental trials.

One of the two treatment rooms was designated for the ELF-EMF exposure treatments, while the other treatment room was designated for the control treatments of the experimental trials. The two experimental trials were conducted sequentially, with Experimental Trial 1 (1-week ELF-EMF exposure) conducted before Experimental Trial 2 (12-weeks ELF-EMF exposure). In the ELF-EMF

exposure treatment room the three treatments were applied concurrently; with the 1-hour and 4-hour treatments commencing at 08:00 daily. The continuous exposure treatment (24-hour treatment) was applied continuously for one week in Experimental Trial 1 and for 12 weeks in Experimental Trial 2.

3.4.2 Mice care

The mice used in this study were cared for according to the specifications of the Animal Unit. These specifications are based upon the instructions described by the Animal Protection Act 71 of 1962 (South-African Goverment 1962). The diet of the mice comprised of specifically formulated food pellets produced by Epol®. Access to food and water was not restricted. Both the feeding pellet toughs and water bottles were replenished daily. The bottom of a mice cage was also covered with pine shavings.

For Experimental Trial 1, the cages were not cleaned during the week of exposure, however, for Experimental Trial 2, all cages were cleaned at the end of all the weeks, except for the last week, at which time the mice were sacrificed. During the cleaning process, disruption of a treatment was kept at a minimum. To ensure minimal disruption, clean cages were prepared beforehand and the exchange for clean cages executed as fast as possible. The mice cages of each PVC cylinder were exchanged for clean cages one cylinder at a time. Firstly, each of the three cages in a PVC cylinder was removed from the cylinder and marked as “1”, “2” and “3”, where “1” indicated the top cage, “2” the middle cage and “3” the bottom cage. Mice were removed from their respective cages and placed into a clean cage with the same number. To establish rotation of the position of cages in the PVC cylinder, the clean cages were then returned to another position in the PVC cylinder in a systematic manner. The clean cages were returned to a PVC cylinder in the order of cage “2” at the bottom, “1” in the middle and “3” on top.

3.4.3 Blood collection

The effect of ELF-EMF was studied on 15 blood parameters. These parameters included four erythrocyte parameters, thrombocytes, six leucocytes parameters and four immunophenotyping cluster of differentiation (CD) classes (Table 3.1).

Table 3.1 Blood parameters assessed for the effect of ELF-EMF.

Erythrocyte and thrombocyte parameters	Leucocyte parameters	CD parameters
Erythrocyte count (RBC)	Total leucocyte count	CD3
Haemoglobin (HGB)	Lymphocyte count	CD4
Haematocrit (HCT)	Monocyte count	CD8
Mean corpuscular volume (MCV)	Neutrophil count	CD19
Thrombocyte count	Eosinophil count	
	Basophil count	

To assess the effect of ELF-EMF on the 15 blood parameters, blood was collected from the mice after the completion of the two trials. The mice cages were taken to the surgery of the Animal Unit one-by-one. Blood was collected from each mouse in a cage as fast as possible to limit any stress. This was achieved by first anaesthetising all the mice from a cage by placing them in a desiccator containing a cotton wool ball drenched with halothane (3-5%). Blood was collected from a mouse through orbital bleeding (Everds and Bollinger 2012; Hoggatt et al. 2016). Pressure was applied to the skull of a mouse to force out an eye, which was then removed with a forceps. Thereafter, approximately 1 ml of blood was collected from the orbital cavity in a blood collecting tube containing ethylenediaminetetraacetic acid (EDTA) anticoagulant powder and gently mixed. At the end of the blood collecting procedure, a mouse had usually died from exsanguination, but not withstanding the neck was dislocated. Each 1 ml blood tube was then gently mixed, after which 0.5 ml of blood was

removed with a syringe and decanted into a cuvette. Both 0.5 ml containers where then marked in an appropriate manner. The blood containing tubes were immediately placed on ice in an icebox and transported to the laboratories where the respective blood parameters were measured within 24 hours. Half of the blood containing cuvettes were sent to the National Health Laboratory Services (NHLS) at the University of the Free State, Bloemfontein, for the CD parameter measurements. The other half of the blood containing cuvettes were sent to the haematology laboratory of the Central University of Technology, Free State, to measure the remainder of the blood parameters.

3.4.4 Blood parameter measurements

The blood parameters, excluding the CD parameters, were analysed with a PENTRA 60C(+) fully automated haematology analyser (Horiba ABS, PENTRA 80, Paris, France). The PENTRA 60C(+) is a small bench-top instrument with single sample capability. It provides a complete blood cell (CBC) count, including a five-part differential (5-DIFF) count, as well as counts of two leucocyte subpopulations; the large immature cells (LIC) and atypical lymphocytes (ALY). Blood samples destined for analysis by the PENTRA 60C(+) were uninterruptedly rotated gently on rollers to prevent coagulation. The PENTRA 60C(+) was operated in the CBC + 5DIFF mode, to include a five-part differential count of the leucocytes. From a particular blood sample, 53 µl of the blood were aspirated with a sampling probe and then divided into three blood samples; which were then distributed to three respective chambers with reagents where the measurements were made (Table 3.2).

Table 3.2 Blood parameters measured in the three different chambers using the CBC+ 5DIFF mode of the PENTRA 60C(+).

Chambers	Parameter	Parameter description
1	WBC	White blood cell
	LYM	Lymphocyte % and number
	MON	Monocyte % and number
	NEU	Neutrophil % and number
	EOS	Eosinophil % and number
	BAS	Basophil % and number
	LIC	Large immature cell % and number
	ALY	Atypical lymphocyte % and number
2	RBC	Red blood cell
	HGB	Haemoglobin concentration
	HCT	Haematocrit
	MCV	Mean corpuscular volume
	MCH	Mean corpuscular haemoglobin
	MCHC	Mean corpuscular haemoglobin concentration
	RDW	Red distribution width
3	PLT	Platelets
	RDW	Platelet distribution width
	MPV	Mean platelet volume
	PCT	Plateletcrit

The blood samples were analysed one-by-one. After analysing a blood sample, a computer generated result was produced of the respective haematological measurements as indicated in Table 3.3.

Table 3.3 Example of a PENTRA 60C(+) Differential mode haematology analyser result.

Parameter	Measurement	Measurement unit	Normal ranges (NR)	
WBC	6.2	$10^3/mm^3$	4.0 / 11.0	
RBC	5.5	$10^6/ mm^3$	4.00 /6.20	
HGB	16.2	g/dl	11.0 /18.8	
HCT	49.6	%	35.0 / 55.0	
MCV	89	μm^3	80 / 100	
MCH*	29.3	pg	26.0 / 34.0	
MCHC*	32.8	g/dl	31.0 / 35.0	
RDW*	21.8 h	%	10.0 / 20.0	
PLT	225	$10^3/mm^3$	150 / 400	
MPV*	9.9	μm^3	6.0 / 10.0	
PCT*	0.22	%	0.200 / 0.500	
PDW*	18.8 h	%	8.0 / 18.0	
	%	**True value $10^3/mm^3$**	**% NR**	**True value NR $10^3/mm^3$**
LYM%	32.7	2.01	25.0 / 50.0	1.00 / 5.00
MON%	5.7	2.0	2.0 / 10.0	0.10 / 1.00
NEU%	58.4	3.60	50.0 / 80.0	2.00 / 8.00
EOS%	1.3	0.08	0.0 / 5.0	0.00 / 0.40
BAS%	1.9	0.12	0.0 / 2.0	0.00 / 0.20
ALY%*	1.4	0.08	0.0 / 2.0	0.00 / 0.20
LIC%*	0.4	0.02	0.0 / 2.0	0.00 / 0.20

* = Blood parameters that were not included in this study; h = Above normal ranges; NR = normal range

The CD blood parameters were analysed with a BD FACSCalibur flow cytometer: This flow cytometer is a multicolour flow cytometry system that performs both analysis and sorting in one benchtop system. The BD FACSCalibur system is a highly sensitive tool, providing laboratories with the technological capabilities required for cellular and molecular work in areas such immune

function, genetics and proteomics. Flow cytometry is a technology that simultaneously measures and then analyses multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence. The argon ion laser is commonly used in flow cytometry, because the 488 nm light that it emits excites more than one fluorochrome. The commonly used fluorochromes, fluorescein isothiocyanate (FITC) and phycoerythrin (PE) were used in this study to label the CD parameters. The combination of the fluorochromes, FITC and PE, can be exited simultaneously, generating peak emission wavelengths at 520 nm for FITC and 575 nm for PE, which are far enough apart so that each signal can be detected by a separate detector. The amount of fluorescent signal detected is proportional to the number of fluorochrome molecules on the particle. For the detection of the CD parameters, CD3, CD4, CD8 and CD19, FITC and PE fluorescent dyes were conjugated to a monoclonal antibody and used to identify a particular cell type based on the individual antigenic surface markers of the cell (Table 3.4). The staining pattern produced by each cell subpopulation, combined with data from forward-scattered light and side-scattered light were used to identify which cells were present in a sample and to count their relative percentages.

Table 3.4 CD parameters, monoclonal antibodies and fluorochromes used in the study.

Parameter	Monoclonal antibody	Fluorochrome	Manufacturer
CD3	IgG2a	FITC	Becton Dickinson
CD4	IgG2b	PE	Becton Dickinson
CD8	IgG2a	PE	Becton Dickinson
CD19	IgG2a	PE	Becton Dickinson
Mouse Control	IgG2a	FITC	Becton Dickinson
Mouse Control	IgG2a	PE	Becton Dickinson
Mouse Control (Clone A-1)	IgG2a	PE	Becton Dickinson

FITC = fluorescein isothiocyanate; PE = phycoerythrin

3.5 Data analysis

Several statistical analyses were performed on the data that were generated in this study. These analyses included the following:

- Summary statistics were calculated for all 15 blood parameters that were measured in Experimental Trial 1 and Experimental Trial 2;
- analysis of variance (ANOVA) tests were performed to ascertain if significant differences existed between treatments of a particular blood parameter within a particular experimental trial;
- Tukey HSD tests were performed after ANOVA tests that were found to be significant. These test were performed to reveal witch combinations of treatments were significantly different from one another; and
- t-tests were performed to ascertain if significant differences existed between equivalent treatments of the two experimental trials.

Chapter 4

Erythrocytes and Thrombocytes

4.1 Introduction

The erythroid lineage is responsible for the formation of erythrocytes, (red blood cells; RBC), and thrombocytes (platelets; PLT). These blood cells originate from a common myeloid progenitor (Figure 4.1). The RBC are responsible for the transport of oxygen to tissue cells, while the platelets are central to this haemostatic balance, support of coagulation, and also show anti-fibrinolytic effects.

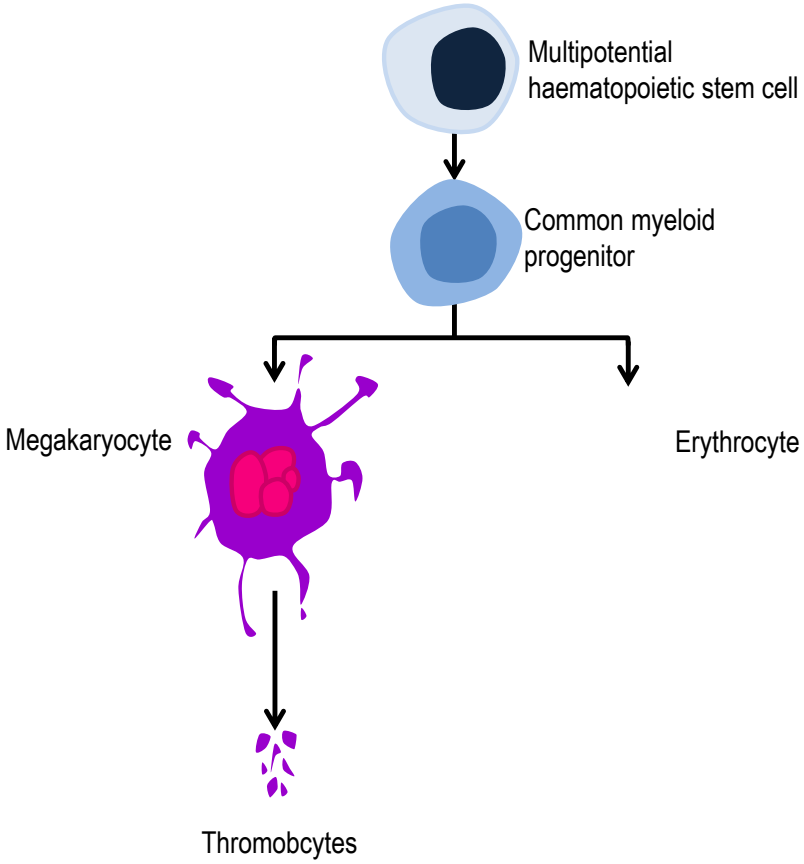


Figure 4.1 Erythrocyte and thrombocyte lineage.

Several studies in rats and mice have shown that ELF-EMF may affect erythrocyte parameters, and thrombocytes. Mice exposed to ELF-EMF for 8 to10 hours/day for of 7, 14, 28, 42 and 56 days, demonstrated decreased numbers of red blood cells and haemoglobin concentrations up to 42 days of exposure (Singh et al. 2013). This study further showed that all changes appeared to normalise during the last two weeks of the 56-day exposure group. Rats exposed for 3 hours/day over 50 and 100 days showed that haemoglobin concentrations and mean platelet volumes (MPV) significantly decreased in rats that were exposed to ELF-EMF for 50 days when compared to a control group (Cakir et al. 2009). When the 50-day exposure group was compared to the 100-day exposure group, it was noticed that MPV levels were significantly lower in the 100-day exposure group. The number of red blood cells, haematocrit and number of platelets did not demonstrate significant differences. Hashish et al. (2008) also showed that in mice exposed continuously for 30 days to ELF-EMF, the number of platelets decreased. In contrast to these studies, Amara et al. (2006) showed that the levels of haemoglobin, red blood cells and number of platelets increased significantly in rats exposed for 1-hour/day for 30 consecutive days. Rats exposed continuously to ELF-EMF for 15, 30 and 45 days showed changes in osmotic fragility and shape of red blood cell membranes and haemoglobin (Ali et al. 2003). These results indicated that exposure of the animals to ELF-EMF resulted in the decrease of red blood cell membrane elasticity and permeability and in changes in the molecular structure of haemoglobin. After the removal from the ELF-EMF, the animals did not show signs of repair in the newly generated red blood cell structure, indicating that the blood generating system was severely impaired.

In this study, mice were exposed to ELF-EMF for one week (Experimental Trial 1) and twelve weeks (Experimental Trial 2). The different treatment groups involved exposure for 1-hour, 4-hours and continuously. The effects of ELF-EMF were measured for five different erythrocyte and thrombocyte parameters and then compared to the excepted normal ranges for mice as listed in Table 4.1.

Table 4.1 Erythrocyte and parameters and normal ranges in mice. (Everds and Bollinger 2012).

Parameter	Unit	Normal mouse range
Erythrocyte count (RBC)	Number of cells × 10^6/mm^3	7–11 × 10^6/mm^3
Haemoglobin concentration (HGB)	Grams per decilitre (g/dl)	13–18 g/dl
Haematocrit (HCT)	Percentage (%)	40–60%
Mean corpuscular volume (MCV)	Femtolitre (fl)	40–55 fl
Thrombocyte count (PLT)	Number of platelets × 10^3/mm^3	900–2000 × 10^3/mm^3

4.2 Experimental Trial 1: One-week exposure

Overall, no distinguishing patterns emerged for the four treatment groups of the different erythrocyte and thrombocyte parameters. The range values were, for the most, similar for each of the treatment groups, however for the parameters RBC, HGB and HCT the largest range values were recorded for the 1-hour and 4-hour ELF-EMF exposure treatment groups (Table 4.2). Besides the PLT, all means of the remaining parameters fell within the normal range for mice (Everds and Bollinger 2012). The PLT measurements were marginally less than the normal range.

Table 4.2 Summary statistics of erythrocyte and thrombocyte parameters of Experimental Trial 1.

Variable	Treatment group	Minimum	Maximum	Range	Mean	Standard deviation
RBC	0-H (control)	8.40	10.30	1.90	9.23	0.49
	1-H	7.70	10.10	2.40	9.21	0.52
	4-H	8.10	10.20	2.10	9.26	0.53
	24-H	8.90	9.90	1.00	9.32	0.28
HGB	0-H (control)	13.60	16.20	2.60	14.75	0.64
	1-H	12.40	15.70	3.30	14.66	0.79
	4-H	12.80	16.10	3.30	14.76	0.71
	24-H	13.90	15.90	2.00	14.76	0.51
HCT	0-H (control)	39.30	48.00	8.70	43.72	2.33
	1-H	37.90	48.10	10.20	43.86	2.45
	4-H	37.50	48.30	10.80	43.88	2.60
	24-H	41.80	46.90	5.10	44.12	1.37
MCV	0-H (control)	46.00	48.00	2.00	47.29	0.55
	1-H	46.00	49.00	3.00	47.60	0.65
	4-H	46.00	48.00	2.00	47.32	0.56
	24-H	47.00	48.00	1.00	47.48	0.51
PLT	0-H (control)	347.00	1,205.00	858.00	853.63	225.15
	1-H	500.00	1,254.00	754.00	883.12	175.44
	4-H	428.00	1,119.00	691.00	837.12	172.52
	24-H	457.00	1,023.00	566.00	824.77	122.14

RBC = Red blood cell count; HGB = Haemoglobin; HCT = Haematocrit; MCV = Mean corpuscular value; PLT = Platelet count; H = Hour

ANOVA tests were performed to ascertain if significant differences existed between the different treatment groups of the different erythrocyte and thrombocyte parameters. All of the ANOVA tests

revealed that no significant differences existed between any of the treatments for all parameters at α = 0.05 (Table 4.3).

Table 4.3 ANOVA tests performed on the erythrocyte and thrombocyte parameters of Experimental Trial 1.

Parameter	Source	Sum of squares	Degrees of freedom	Mean square	*F*	*P*-value
RBC	Between groups	0.15	3	0.05	0.23	0.88
	Within groups	20.65	95	0.22		
	Total	20.80				
HGB	Between groups	0.17	3	0.06	0.13	0.94
	Within groups	42.84	95	0.45		
	Total	43.01	98			
HCT	Between groups	2.04	3	0.68	0.14	0.94
	Within groups	476.75	95	5.02		
	Total	478.81	98			
MCV	Between groups	81.07	3	27.02	1.19	0.32
	Within groups	2,177.68	96	22.68		
	Total	2,258.75	99			
PLT	Between groups	50,337.24	3	16,779.08	0.44	0.72
	Within groups	3,609,366.00	95	37,993.33		
	Total	3,659,703.45	98			

RBC = Red blood cell count; HGB = Haemoglobin; HCT = Haematocrit; MCV = Mean corpuscular value; PLT = Platelet count

4.3 Experimental Trial 2: Twelve-week exposure

In contrast with the Experimental Trial 1, a few distinguishing patterns could be discerned for the different treatment groups of the different erythrocyte and thrombocyte parameters measured in Experimental Trial 2. The range values of RBC, HGB and HCT demonstrated a tendency to increase towards the higher ELF-EMF exposure times (Table 4.4). The range values of the 1-hour and 4-hour ELF-EMF treatment groups for HCT were approximately double that of the control treatment group, while the range value of the 24-hour ELF-EMF treatment group was approximately four times greater than that of the control treatment group. In contrast for MCV, a decrease in range values was noted for the higher ELF-EMF exposure times. For all erythrocyte and thrombocyte parameters, except for PLT, the mean values were relatively similar for all treatment groups. For the PLT, the mean number of PLT demonstrated a decrease with an increase in the ELF-EMF exposure times. Besides the PLT, the means of the remaining parameters fell within the normal range for mice (Everds and Bollinger 2012). The PLT measurements were marginally less than the normal range.

Table 4.4 Summary statistics of erythrocyte and thrombocyte parameters of Experimental Trial 2.

Variable	Treatment group	Minimum	Maximum	Range	Mean	Standard deviation
RBC	0-H (control)	8.17	10.10	1.93	9.21	0.59
	1-H	7.33	10.49	3.16	9.27	0.68
	4-H	7.56	11.06	3.50	9.48	0.83
	24-H	3.38	11.01	7.63	9.23	1.38
HGB	0-H (control)	13.50	16.60	3.10	14.77	0.85
	1-H	13.00	16.30	3.30	14.68	0.90
	4-H	12.00	17.50	5.50	15.05	1.34
	24-H	5.30	17.20	11.90	14.54	2.16

Variable	Treatment group	Minimum	Maximum	Range	Mean	Standard deviation
HCT	0-H (control)	39.70	48.40	8.70	44.08	2.63
	1-H	34.10	49.70	15.60	43.63	3.33
	4-H	35.40	52.60	17.20	44.98	4.20
	24-H	15.60	54.00	38.40	43.83	6.83
MCV	0-H (control)	46.00	55.00	9.00	47.88	1.99
	1-H	46.00	48.00	2.00	47.08	0.58
	4-H	47.00	48.00	1.00	47.48	0.51
	24-H	44.30	48.00	3.70	46.85	0.77
PLT	0-H (control)	325	983	658	813.63	157.36
	1-H	254	1,115	861	803.13	193.33
	4-H	399	1,024	625	771.60	171.00
	24-H	383	1,094	711	708.83	191.05

RBC = Red blood cell count; HGB = Haemoglobin; HCT = Haematocrit; MCV = Mean corpuscular value; PLT = Platelet; H = Hour

ANOVA tests were performed to ascertain if significant differences existed between the different treatment groups of the different erythrocyte and thrombocyte parameters measured in Experimental Trial 2. Highly significant differences could only be identified for the parameter MCV at $\alpha = 0.05$ (Table 4.5). All other parameters did not reveal significant differences.

Table 4.5 **ANOVA tests performed on the erythrocyte and thrombocyte parameters of Experimental Trial 2.**

Parameter	Source	Sum of squares	Degrees of freedom	Mean square	*F*	*P*-value
RBC	Between groups	1.11	3	0.73	0.41	0.74
	Within groups	76.25	85	0.89		
	Total	77.36	88			
HGB	Between groups	3.51	3	1.17	0.56	0.64
	Within groups	180.13	86	2.09		
	Total	183.64	89			
HCT	Between groups	28.00	3	9.33	0.43	0.76
	Within groups	1,859.52	86	21.62		
	Total	1,887.52	89			
MCV	Between groups	11.99	3	3.99	3.93	**0.01**
	Within groups	87.71	86	1.01		
	Total	99.71	89			
PLT	Between groups	146,529.06	3	48,843.02	1.52	0.22
	Within groups	2,735,022.92	85	32,176.74		
	Total	2,881,551.97	88			

*RBC = Red blood cell count; HGB = Haemoglobin; HCT = Haematocrit; MCV = Mean corpuscular value; PLT = Platelet count

A Tukey HSD test was performed on the MCV data to determine which of the different treatment pairs differed from one another at $\alpha = 0.05$. This test revealed that the control treatment and 24-hour exposure treatment groups demonstrated highly significant differences ($\boldsymbol{p} = 0.01$).

4.4 Comparison of Experimental Trial 1 and Experimental Trial 2

Most of the ranges (70%) of the measurements of the erythrocyte and thrombocyte parameters were larger (wider) in Experimental Trial 2, when compared to Experimental Trial 1. In contrast, only 45%

of the means of the erythrocyte and thrombocyte parameters of Experimental Trial 2 were greater than the means of Experimental Trial 1 (Table 4.6). For the parameters RBC number, HGB and HCT; the smallest differences between the ranges of Experimental Trial 1 and 2 were recorded for the control treatment groups and the highest differences between ranges for the 24-hour treatment groups. In contrast, the largest differences between ranges were recorded for the control treatment groups for the parameters MCV and PLT. The 24-hour treatment group of HCT showed an exceptionally high value for the difference between the ranges, which was more than five times greater than that of the 1-hour and 4-hour treatment groups. The largest differences between means of Experimental Trial 1 and 2 were recorded for the 4-hour treatment groups of RBC, HGB and HCT, while for MCV and PLT the 24-hour treatment groups demonstrated the largest differences between means.

Table 4.6 Comparison of ranges and means between Experimental Trials 1 and 2.

Variable	Treatment group	Range Exp. 1	Mean Exp. 1	Range Exp. 2	Mean Exp. 2	Difference between ranges	Difference between means
RBC	0-H (control)	1.90	9.23	1.93	9.21	-0.03	0.02
	1-H	2.40	9.21	3.16	9.27	-0.76	-0.06
	4-H	2.10	9.26	3.50	9.48	-1.40	-0.22
	24-H	1.00	9.32	7.63	9.23	-6.60	0.09
HGB	0-H (control)	2.60	14.75	3.10	14.77	-0.50	-0.02
	1-H	3.30	14.66	3.30	14.68	0.00	-0.02
	4-H	3.30	14.76	5.50	15.05	-2.20	-0.29
	24-H	2.00	14.76	11.90	14.54	-9.90	0.22
HCT	0-H (control)	8.70	43.72	8.70	44.08	0.00	-0.36
	1-H	10.20	43.86	15.60	43.63	-5.40	0.23
	4-H	10.80	43.88	17.20	44.98	-6.40	-1.1
	24-H	5.10	44.12	38.40	43.83	-33.30	0.29

Variable	Treatment group	Range Exp. 1	Mean Exp. 1	Range Exp. 2	Mean Exp. 2	Difference between ranges	Difference between means
MCV	0-H (control)	2.00	47.29	9.00	47.88	-7.00	-0.59
	1-H	3.00	47.60	2.00	47.08	1.00	0.52
	4-H	2.00	47.32	1.00	47.48	1.00	-0.16
	24-H	1.00	47.48	3.70	46.85	-2.70	0.63
PLT	0-H (control)	858.00	853.63	658	813.63	200	40
	1-H	754.00	883.12	861	803.13	-107	79.99
	4-H	691.00	837.12	625	771.60	66	65.52
	24-H	566.00	824.77	711	708.83	-145	115.94

RBC = Red blood cell count; HGB = Haemoglobin; HCT = Haematocrit; MCV = Mean corpuscular value; PLT = Platelet count; H = Hour; Exp. 1 = Experimental Trial 1; Exp. 2 = Experimental Trial 2

T-tests were performed to ascertain if the respective treatments of a particular parameter in Experimental Trial 1 differed significantly from the equivalent treatment in Experimental Trial 2. These t-tests revealed that a few of the comparisons were significantly different at $\alpha = 0.05$ (Table 4.7). For the parameter HGB, all the ELF-EMF exposure treatment groups (1-hour, 4-hour and 24-hour) showed highly significant differences between the two experimental trials. For the number of PLT, only the 24-hour treatment group was highly significant. The parameter MCV revealed highly significant differences between the 4-hour treatment groups, as well as between the 24-hour treatment groups of the two experimental trials.

Table 4.7 **T-tests comparing the erythrocyte and thrombocyte parameters of Experimental Trials 1 and 2.**

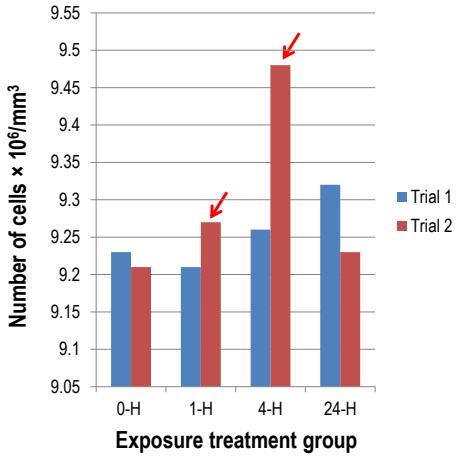
Parameter	Treatment group	df	t	*P*-value
RBC	0-H	28	0.12	0.90
	1-H	45	-0.27	0.78
	4-H	41	-1.12	0.27
	24-H	25	0.30	0.76
HGB	0-H	26	-0.09	0.92
	1-H	25	-41.44	**< 0.0001**
	4-H	25	-35.45	**< 0.0001**
	24-H	25	-20.76	**< 0.0001**
HCT	0-H	29	-0.43	0.66
	1-H	42	0.31	0.76
	4-H	40	-1.11	0.27
	24-H	25	0.20	0.83
MCV	0-H	17	-1.14	0.27
	1-H	47	2.94	**< 0.01**
	4-H	48	-1.06	0.29
	24-H	40	3.38	**< 0.001**
PLT	0-H	38	0.66	0.51
	1-H	45	1.49	0.14
	4-H	48	1.35	0.18
	24-H	39	3.02	**< 0.001**

RBC = Red blood cell count; HGB = Haemoglobin; HCT = Haematocrit;
MCV = Mean corpuscular value; PLT = Platelet count; H = Hour

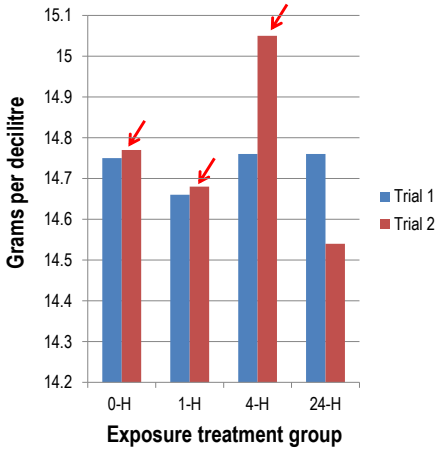
A visual perspective of the mean percentages of the respective exposure treatment groups of the two experimental trials shows a number of identifiable differences. Parameters RBC, HGB, HCT and MCV demonstrated lower mean values for the 4-hour treatment group in Experimental Trial 1 when compared to the 4-hour groups of Experimental Trial 2 (Figure 4.2). In contrast, for the same

parameters, the 24-hour treatment group demonstrated higher mean values in Experimental Trial 1 when compared to those of Experimental Trial 2. When considering the control treatment groups of HGB, HCT and MCV, the mean values in Experimental Trial 1 were lower than those of Experimental Trial 2. For the PLT count, the Experimental Trial 1 mean values were all greater than the Experimental Trial 2 mean values.

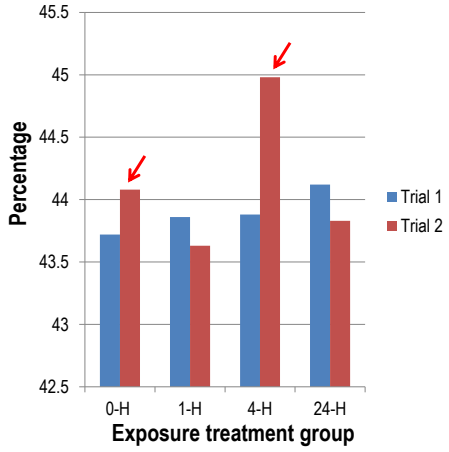
a. RBC



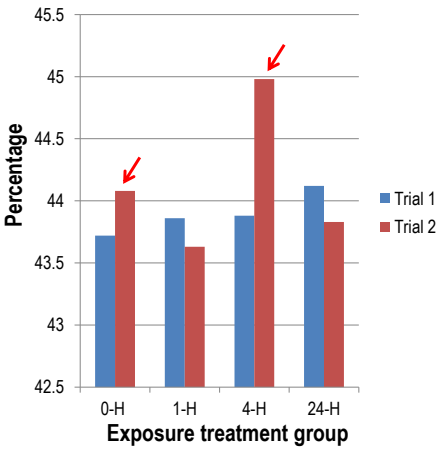
b. HGB



c. HCT



d. MCV



e. PLT

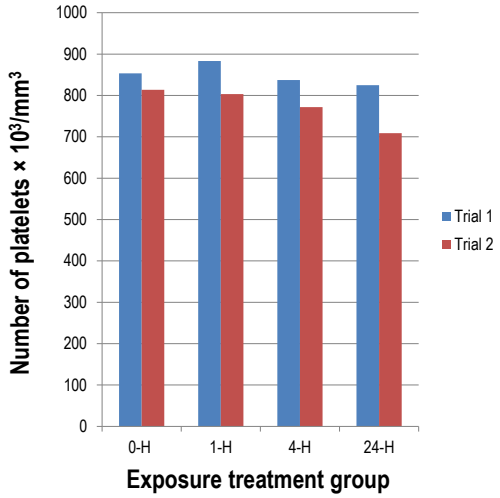


Figure 4.2 Bar graph of the comparison of Experimental Trial 1 and 2 mean values of the different RBC and PLT parameters.

4.5 Discussion

This study showed that ELF-EMF had relatively negligible effects on the erythrocyte and thrombocyte parameters of mice exposed to ELF-EMF for one week, as well as for twelve weeks. MCV was the only parameter that showed significant differences between the control treatment group and the 24-hour treatment group in the 12-week experimental trial. This result indicated that the mean volume of the red blood cells deceased, although slightly, during the 12-week experimental trial.

The results suggest that ELF-EMF may affect red blood cells in various ways. Hashish et al. (2008) demonstrated that ELF-EMF caused a decrease in the number of red blood cells in mice, which was similar to what Amara et al. (2006) showed in rats after a lengthy period of ELF-EMF exposure. Furthermore, Ali et al. (2003) suggested that ELF-EMF caused changes in red blood cell membrane structure and permeability, which could explain the results found for MCV. Little changes could be demonstrated in the number of thrombocytes, which is in contrast to the reduction in the number of thrombocytes shown by Hashish et al. (2008) in mice and Cakir et al. (2009) in rats. Furthermore, the mean values of all parameters of both experimental trials were within the normal ranges for mice,

except for platelets (Everds and Bollinger 2012). In both experimental trials, the mean number of platelets was for all treatments marginally less than the minimum value of the normal range of the number of platelets for mice (Everds and Bollinger 2012); although all treatment groups of Experimental Trial 1 demonstrated slightly higher numbers of platelets when compared to Experimental Trial 2.

A noteworthy outcome of this study was the number of significant differences recorded between the two experimental trials. Although only MCV demonstrated significant differences between ELF-EMF treatments in Experimental Trial 2, a number of significant differences were recorded for ELF-EMF treatments between the two experiments. These included HGB (1-hour, 4-hour and 24-hour treatments), MCV (1-hour and 24-hour treatments) and platelets (24-hour treatment). It is perhaps reasonable to speculate that the longer exposure in Experimental Trial 2 could have induced subclinical stress in the animals. Szemerszky et al. (2010) showed significant proopiomelanocortin elevation and depressive-like behaviour in rats following long-term ELF-EMF exposure, indicating that ELF-EMF may induce a subclinical stress condition.

Chapter 5

Leucocytes

5.1 Introduction

Leucocytes, also known as white blood cells, are major roll players in the immune system. These cells are all nucleated, which distinguishes them from the anucleated erythrocytes and thrombocytes. They are involved in protecting the body against both infectious diseases and foreign invaders (Marieb and Hoehn 2016). Broadly, the leucocytes can be further divided into the five main cell types: neutrophils, eosinophils, basophils, monocytes and lymphocytes (Martini 2004).

When leucocytes are classified by their structure, there are two categories; namely, the granulocytes and agranulocytes (Hall and Guyton 2011). The granulocytes are polymorphonulcear leucocytes and comprise of neutrophils, eosinophils and basophils, while the agranulocytes are mononulcear and comprise of monocytes and lymphocytes. Alternatively, the leucocytes can be classified by their lineage, as either being myeloid or lymphoid cells (Figure 5.1). The lymphocytes form the lymphoid cell lineage, while all the other leucocytes belong to the myeloid cell lineage (Hall and Guyton 2011).

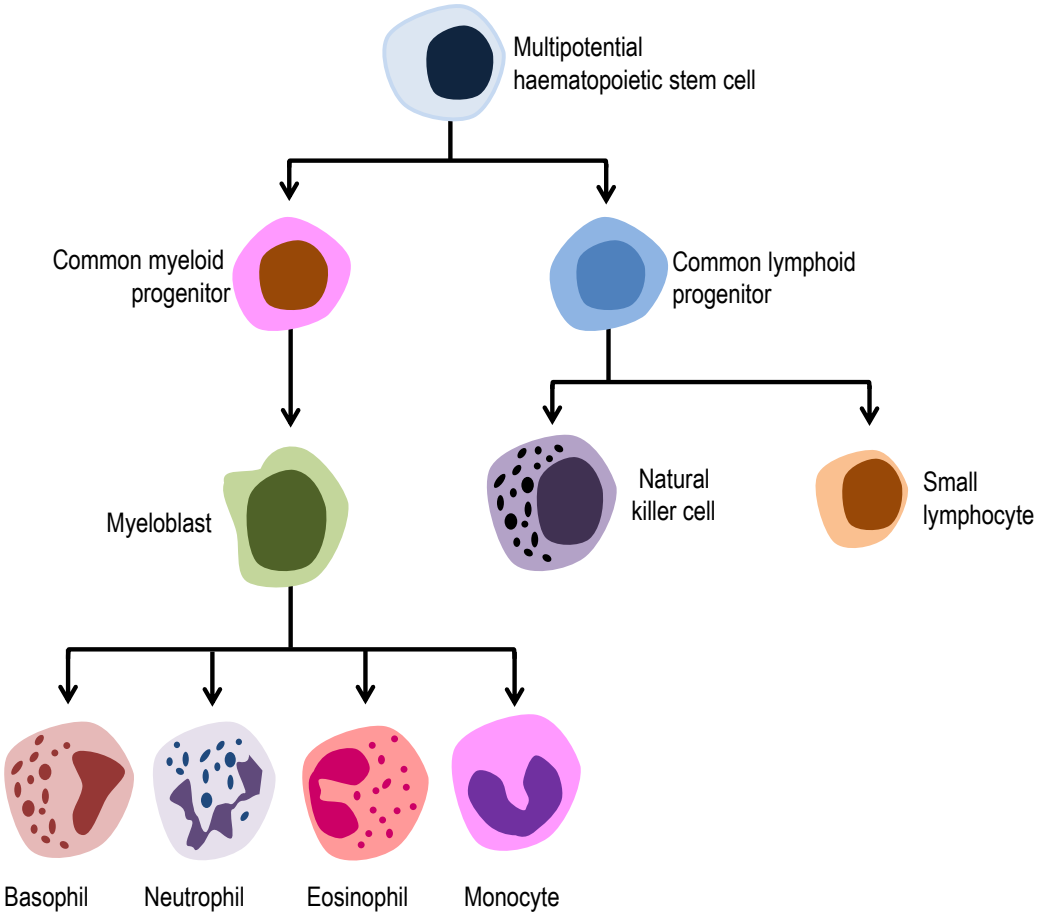


Figure 5.1 Leucocyte lineage.

Several studies in rats and mice have shown that ELF-EMF may affect leucocyte parameters. Studies where rats and mice were exposed continuously to ELF-EMF for one week, 15 weeks (de Kleijn et al. 2016) and for 120 days (Seto et al. 1986) revealed contradicting outcomes for the total number of leucocytes. In a one-week experiment, de Kleijn et al. (2016) demonstrated an increase in the number of leucocytes, while in a 15-week experiment no changes were demonstrated. Seto et al. (1986), on the other hand, showed a decrease in the number of leucocytes.

Where rats and mice were exposed to ELF-EMF for short periods in a day, no particular patterns could be discerned for the respective leucocyte parameters. In a rat experiment where rats were

exposed for 3h/d for 50 and 100 days, no changes could be identified in the number of total leucocytes, as well as in the number of granulocytes and agranulocytes (Cakir et al. 2009). In a number of mice experiments, where mice were exposed to ELF-EMF for short periods in a day over varying number of days, contrasting results were demonstrated. In experiments were mice were exposed for 8 to10 hours/day for 7, 14, 28 and 42 days; Singh et al. (2012) showed an increase in the number of leucocytes and lymphocytes, while the number of monocytes and neutrophils decreased for all exposure periods. In contrast, Çetin et al. (2006) showed opposite results for the total number of leucocytes, lymphocytes and neutrophils. The number of neutrophils decreased similarly, to what Singh et al. (2012) had found. In an experiment where mice were exposed to ELF-EMF for 30 minutes/day every other day for two weeks, the total number of monocytes decreased (Arafa et al. 2003).

In a unique experiment, Bonhomme-Faivre et al. (1998a) exposed Swiss mice continuously to ELF-EMF for 350 days. A number of haematological parameters was measured after 20, 43, 63, 90, 190 and 350 days. On day 20, exposed animals showed a significant decrease in leucocyte, lymphocyte and monocyte counts. On days 43 and 63, no significant differences were observed in the leucocyte parameters, indicating haemopoetic recovery. On day 90, a significant fall in the total number of leucocytes, neutrophils and eosinophils was observed. On day 190, the exposed animals demonstrated neutropenia, while on day 350, no significant differences in the leucocyte parameters were observed, indicating that the animals had recovered.

The effects of ELF-EMF were studied on six different leucocyte parameters in mice exposed for one week (Experimental Trial 1) and for twelve weeks (Experimental Trial 2). The results of the two experiments were compared with one another and to the excepted normal ranges of different leucocyte parameters in mice (Table 5.1).

Table 5.1 Leucocyte parameters and normal ranges in mice (Everds and Bollinger 2012).

Parameter	Unit	Normal mouse range
Total leucocyte count	Number of cells $\times 10^3/mm^3$	$2–10 \times 10^3/mm^3$
Lymphocyte count	Number of cells $\times 10^3/mm^3$	$7–8 \times 10^3/mm^3$
Monocyte count	Number of cells $\times 10^3/mm^3$	Minor cell type
Neutrophil count	Number of cells $\times 10^3/mm^3$	$2–3 \times 10^3/mm^3$
Eosinophil count	Number of cells $\times 10^3/mm^3$	Minor cell type
Basophil count	Number of cells $\times 10^3/mm^3$	Very rarely observed in peripheral blood

5.2 Experimental Trial 1: One-week exposure

Overall, a few distinguishing patterns emerged for the four treatment groups of the different leucocyte parameters. The range values were, for the most, similar for each of the treatment groups of total leucocytes, lymphocytes and basophils. The range values of the different treatment groups of monocytes demonstrated a tendency to increase towards the longer ELF-EMF exposure times. In contrast for the neutrophils, a decrease in range values was noted for the longer ELF-EMF exposure periods (Table 5.2). The mean values demonstrated a slight increase towards the longer ELF-EMF exposure times for total leucocytes, lymphocytes and eosinophils. The mean values of the neutrophils were substantially lower in the ELF-EMF exposure groups when compared to the control treatment group. Besides neutrophils, all the means of the measurements of the remaining parameters fell within the normal range for mice (Everds and Bollinger 2012). The neutrophil measurements were substantially less than the normal range for all treatment groups.

Table 5.2 Summary statistics of leucocyte parameters of Experimental Trial1.

Variable	Treatment group	Minimum	Maximum	Range	Mean	Standard deviation
Total leucocytes	0-H (control)	3.20	9.00	5.80	6.33	1.39
	1-H	4.10	10.90	6.80	6.66	1.43
	4-H	4.46	9.50	4.90	7.06	1.44
	24-H	3.38	11.60	7.80	7.26	1.66
Lymphocytes	0-H (control)	2.69	8.02	5.33	5.51	1.27
	1-H	3.50	9.39	5.90	5.83	1.33
	4-H	3.87	8.68	4.81	6.20	1.37
	24-H	3.34	9.21	5.87	6.49	1.43
Monocytes	0-H (control)	0.06	0.37	0.32	0.20	0.09
	1-H	0.06	0.50	0.44	0.22	0.11
	4-H	0.09	0.79	0.70	0.23	0 .14
	24-H	0.06	1.58	1.52	0.23	0.29
Neutrophils	0-H (control)	0.32	0.93	0.61	0.57	0.15
	1-H	0.02	0.05	0.04	0.03	0.08
	4-H	0.02	0.05	0.03	0.03	0.07
	24-H	0.00	0.08	0.08	0.03	0.02
Eosinophils	0-H (control)	0.00	0.07	0.07	0.02	0.02
	1-H	0.35	0.88	0.54	0.57	0.14
	4-H	0.00	1.02	1.02	0.57	0.19
	24-H	0.23	0.78	0.55	0.49	0.18
Basophils	0-H (control)	0.01	0.04	0.03	0.03	0.01
	1-H	0.00	0.07	0.07	0.02	0.02
	4-H	0.00	0.08	0.08	0.02	0.02
	24-H	0.00	0.07	0.07	0.01	0.02

H = hour

To provide a visual perspective of the outcomes of the different ELF-EMF exposure treatment groups of the respective leucocyte parameters, a bar graph was constructed. The bar graph clearly demonstrates an increase in the total number of leucocytes, lymphocytes and eosinophils (Figure 5.2). The total number of neutrophils was substantially higher in the control treatment group when compared to the ELF-EMF exposure treatment groups. The number of basophils appeared to remain relatively low in all treatment groups.

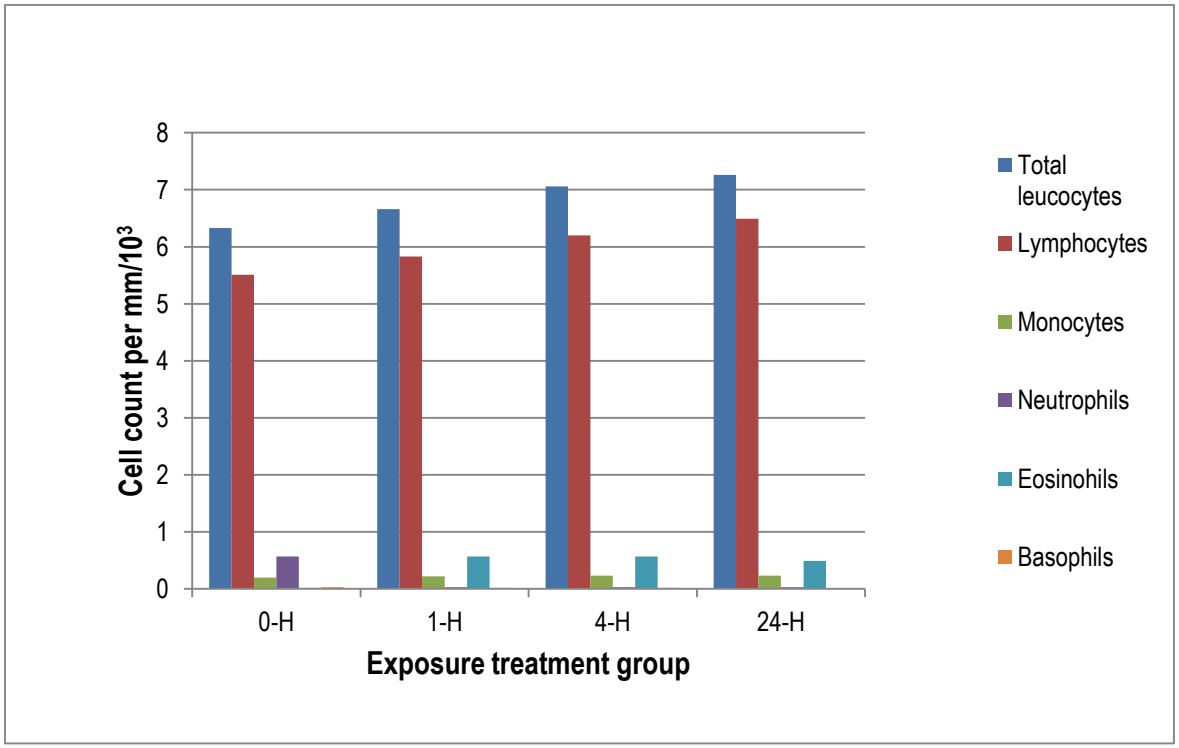


Figure 5.2 **Bar graph showing the mean values of the different leucocyte parameter treatments for Experimental Trial 1.**

ANOVA tests were performed to ascertain if significant differences existed between the different treatment groups of the different leucocyte parameters. The ANOVA tests revealed highly significant differences amongst the treatment groups for the number of neutrophils, eosinophils and basophils (Table 5.3).

Table 5.3 ANOVA tests performed on the leucocyte parameters of Experimental Trial 1.

Parameter	Source	Sum of squares	Degrees of freedom	Mean square	*F*	*P*-value
Total Leucocytes	Between groups	12,623,290.91	3	4,207,763.64	1.91	0.13
	Within groups	209,445,800.00	95	2,204,692.63		
	Total	222,069,090.90	98			
Lymphocytes	Between groups	13,582,784.12	3	4,527,594.71	2.48	0.07
	Within groups	173,446,103.23	95	1,825,748.46		
	Total	187,028,887.35	98			
Monocytes	Between groups	11,816.98	3	3,938.99	0.13	0.94
	Within groups	2,988,472.48	95	31,457.61		
	Total	3,000,289.46	98			
Neutrophils	Between groups	5,249,030.02	3	1,749,676.67	334.82	**< 0.0001**
	Within groups	496,437.20	95	5,225.65		
	Total	5,745,467.22	98			
Eosinophils	Between groups	4,817,880.42	3	1,605,960.14	78.30	**< 0.0001**
	Within groups	1,866,440.30	91	20,510.33		
	Total	6,684,320.72	94			
Basophils	Between groups	22,723.42	3	7,574.47	15.41	**< 0.0001**
	Within groups	41,257.83	84	491.38		
	Total	63,999.25	87			

Tukey HSD tests were performed to determine which pairs of treatment groups were significantly different at $\alpha = 0.05$ for total number of neutrophils, eosinophils and basophils. Significant differences were found between the control treatment groups and all the ELF-EMF exposure treatment groups for the total number of neutrophils and eosinophils (Table 5.4). In contrast, for the basophils, significant differences were found between the control treatment group and the 1-hour treatment group; as well as between the 1-hour treatment group and the 4-hour and 24-hour treatment groups.

Table 5.4 Tukey HSD tests for number of neutrophils, eosinophils and basophils.

Neutrophils				
TG	**0-H**	**1-H**	**4-H**	**24-H**
0-H				
1-H	**S**			
4-H	**S**	NS		
24-H	**S**	NS	NS	

Eosinophils				
TG	**0-H**	**1-H**	**4-H**	**24-H**
0-H				
1-H	**S**			
4-H	**S**	NS		
24-H	**S**	NS	NS	

Basophils				
TG	**0-H**	**1-H**	**4-H**	**24-H**
0-H				
1-H	**S**			
4-H	NS	**S**		
24-H	NS	**S**	NS	

S = significant; NS = non-significant; TG = treatment group; 0-H = control, 1-H = 1 hour, 4-H = 4 hours, 24-H = 24 hours

5.3 Experimental Trial 2: Twelve-week exposure

Similarly, to Experimental Trial 1, a number of distinguishing patterns could be discerned for the different treatment groups of the different leucocyte parameters measured in Experimental Trial 2. For the most, the range values determined in Experimental Trial 2 were similar to those of Experimental Trial 1, except for the number of monocytes for which the range values were substantially less when compared to Experimental Trial 1 (Table 5.5). Similarly, to Experimental Trial 1, the mean values for the three parameters, total number of leucocytes, lymphocytes and eosinophils, were consistently lowest in the control treatment group. In contrast to Experimental Trial 1, the mean number of monocytes was substantially lower in all treatment groups. Furthermore, the number of neutrophils was similar in all treatment groups, which was in contrast to Experimental Trial 1 where the control treatment group showed a relatively high number of neutrophils. Similarly, to the Experimental Trial 1, the number of basophils was exceptionally low in all treatment groups.

Table 5.5 Summary statistics of leucocyte parameters of Experimental Trial 2.

Variable	Treatment group	Minimum	Maximum	Range	Mean	Standard deviation
Total leucocytes	0-H (control)	0.20	7.00	6.80	3.68	2.31
	1-H	2.20	6.40	4.20	4.07	1.34
	4-H	2.70	7.90	5.20	4.53	1.37
	24-H	1.10	8.00	6.90	5.43	1.71
Lymphocytes	0-H (control)	0.07	6.27	6.20	3.14	2.23
	1-H	1.74	5.80	4.13	3.67	1.28
	4-H	2.35	6.72	4.37	4.07	1.20
	24-H	1.02	7.21	6.19	4.98	1.59
Monocytes	0-H (control)	0.00	0.15	0.15	0.06	0.05
	1-H	0.01	0.12	0.11	0.06	0.03
	4-H	0.03	0.17	0.14	0.09	0.04
	24-H	0.02	0.14	0.12	0.09	0.03
Neutrophils	0-H (control)	0.00	0.08	0.08	0.01	0.02
	1-H	0.01	0.35	0.34	0.04	0.09
	4-H	0.00	0.04	0.04	0.01	0.01
	24-H	0.00	0.03	0.03	0.02	0.01
Eosinophils	0-H (control)	0.00	0.57	0.57	0.23	0.17
	1-H	0.17	0.47	0.30	0.30	0.09
	4-H	0.16	0.77	0.61	0.35	0.17
	24-H	0.06	0.59	0.53	0.34	0.12
Basophils	0-H (control)	0.00	0.02	0.02	0.01	0.01
	1-H	0.00	0.02	0.02	0.05	0.05
	4-H	0.00	0.21	0.21	0.02	0.05
	24-H	0.00	0.03	0.03	0.01	0.01

H = hour

To provide a visual perspective of the outcomes of the different ELF-EMF exposure treatment groups of the respective leucocyte parameters, a bar graph was constructed. The bar graph demonstrates the gradual increase in the total number of leucocytes and lymphocytes from the control treatment group to the 24-hour ELF-EMF exposure treatment groups (Figure 5.2). The remainder of the leucocyte parameters showed relatively similar numbers in the respective treatment groups.

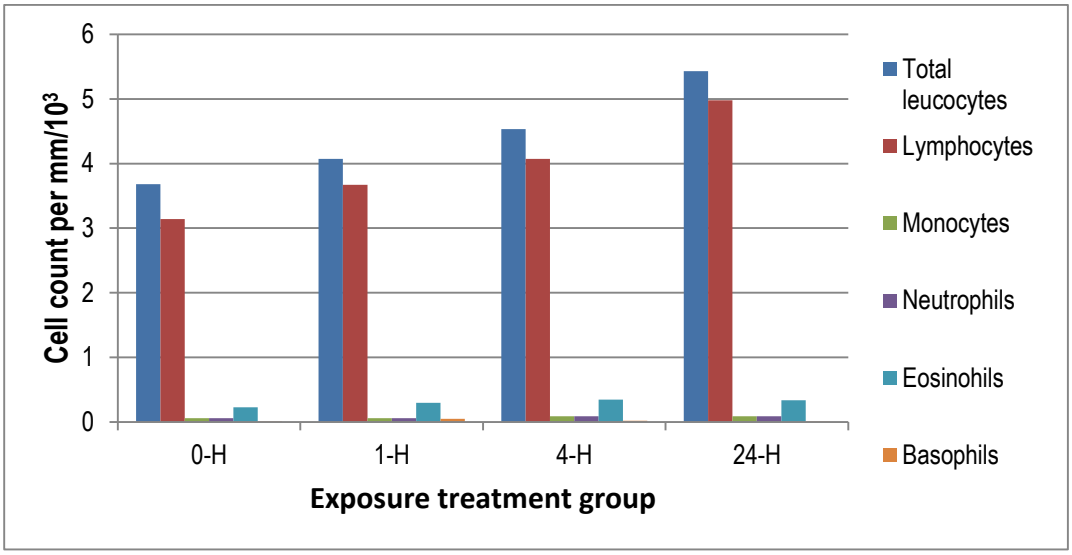


Figure 5.3 **Bar graph showing the mean values of the different leucocyte parameter treatments for Experimental Trial 2.**

Analysis of variance (ANOVA) tests were performed to ascertain if significant differences existed amongst the different treatment groups of the different leucocyte parameters at $\alpha = 0.05$. All of the ANOVA tests revealed that no significant differences existed between any of the treatments for all parameters at $\alpha = 0.05$ (Table 5.6).

Table 5.6 **ANOVA tests performed on the erythrocyte and thrombocyte parameters of Experimental Trial 2.**

Parameter	Source	Sum of squares	Degrees of freedom	Mean square	*F*	*P*-value
Total leucocytes	Between groups	18,960,507.94	3	6,320,169.31	1.28	0.27
	Within groups	344,299,492.06	70	4,918,564.17		
	Total	363,260,000.00	73			
Lymphocytes	Between groups	19,255,581.85	3	6,418,527.28	1.51	0.22
	Within groups	297,901,366.23	70	4,255,733.80		
	Total	317,156,948.08	73			
Monocytes	Between groups	6,815.89	3	2,271.96	1.26	0.29
	Within groups	126,039.63	70	1,800.57		
	Total	132,855.52	73			
Neutrophils	Between groups	4,272.95	3	1,424.32	0.69	0.56
	Within groups	111,266.55	54	2,060.49		
	Total	115539.51	57			
Eosinophils	Between groups	36,067.63	3	12,022.54	0.42	0.74
	Within groups	1,983,325.39	70	28,333.22		
	Total	2,019,393.02	73			
Basophils	Between groups	1,451.39	3	483.80	0.63	0.60
	Within groups	39,316.71	51	770.92		
	Total	40,768.10	54			

5.4 Comparison of Experimental Trial 1 and Experimental Trial 2

When the range values of the two experimental trials were compared, no distinguishing patterns could be identified. However, when the means of Experimental Trial 1 were compared to the means of Experimental Trial 2, it was noted that 79% of the means of the different treatment groups were greater in Experimental Trial 1 than those in Experimental Trial 2 (Table 5.7). For the parameters total number of leucocytes, lymphocytes and monocytes, the mean values of all the treatment

groups were greater in Experimental Trial 1, when compared to Experimental Trial 2. The control treatment group of the total number of neutrophils measured in Experimental Trial 1 was approximately fifty times greater than Experimental Trial 2, while all the measurements of the ELF-EMF exposure treatment groups were relatively similar in the two trials. On the other hand, the control treatment group of the total number of eosinophils measured in Experimental Trial 2 was approximately ten times greater than Experimental Trial 1, while all the measurements of the ELF-EMF exposure treatment groups were relatively similar in the two trials.

Table 5.7 Comparison of ranges and means between Experimental Trials 1 and 2.

Variable	Treatment group	Range Exp. 1	Mean Exp. 1	Range Exp. 2	Mean Exp. 2	Difference in ranges	Difference in means
Total leucocytes	0-H (control)	5.80	6.33	6.80	3.68	-1.00	2.65
	1-H	6.80	6.66	4.20	4.07	2.60	2.59
	4-H	4.90	7.06	5.20	4.53	-0.30	2.53
	24-H	7.80	7.26	6.90	5.43	0.90	1.83
Lymphocytes	0-H (control)	5.33	5.51	6.20	3.14	-0.87	2.37
	1-H	5.90	5.83	4.13	3.67	1.77	2.16
	4-H	4.81	6.20	4.37	4.07	0.44	2.13
	24-H	5.87	6.49	6.19	4.98	-0.32	1.51
Monocytes	0-H (control)	0.32	0.20	0.15	0.06	0.17	0.14
	1-H	0.44	0.22	0.11	0.06	0.33	0.16
	4-H	0.70	0.23	0.14	0.09	0.56	0.14
	24-H	1.52	0.23	0.12	0.09	1.40	0.14

Variable	Treatment group	Range Exp. 1	Mean Exp. 1	Range Exp. 2	Mean Exp. 2	Difference in ranges	Difference in means
Neutrophils	0-H (control)	0.61	0.57	0.08	0.01	0.53	0.56
	1-H	0.04	0.03	0.34	0.04	-0.30	-0.01
	4-H	0.03	0.03	0.04	0.01	-0.01	0.02
	24-H	0.08	0.03	0.03	0.02	0.05	0.01
Eosinophils	0-H (control)	0.07	0.02	0.57	0.23	-0.5	-0.21
	1-H	0.54	0.57	0.30	0.30	0.24	0.27
	4-H	1.02	0.57	0.61	0.35	0.41	0.22
	24-H	0.55	0.49	0.53	0.34	0.02	0.15
Basophils	0-H (control)	0.03	0.03	0.02	0.01	0.01	0.02
	1-H	0.07	0.02	0.02	0.05	0.05	-0.03
	4-H	0.08	0.02	0.21	0.02	-0.13	0.00
	24-H	0.07	0.01	0.03	0.01	0.04	0.00

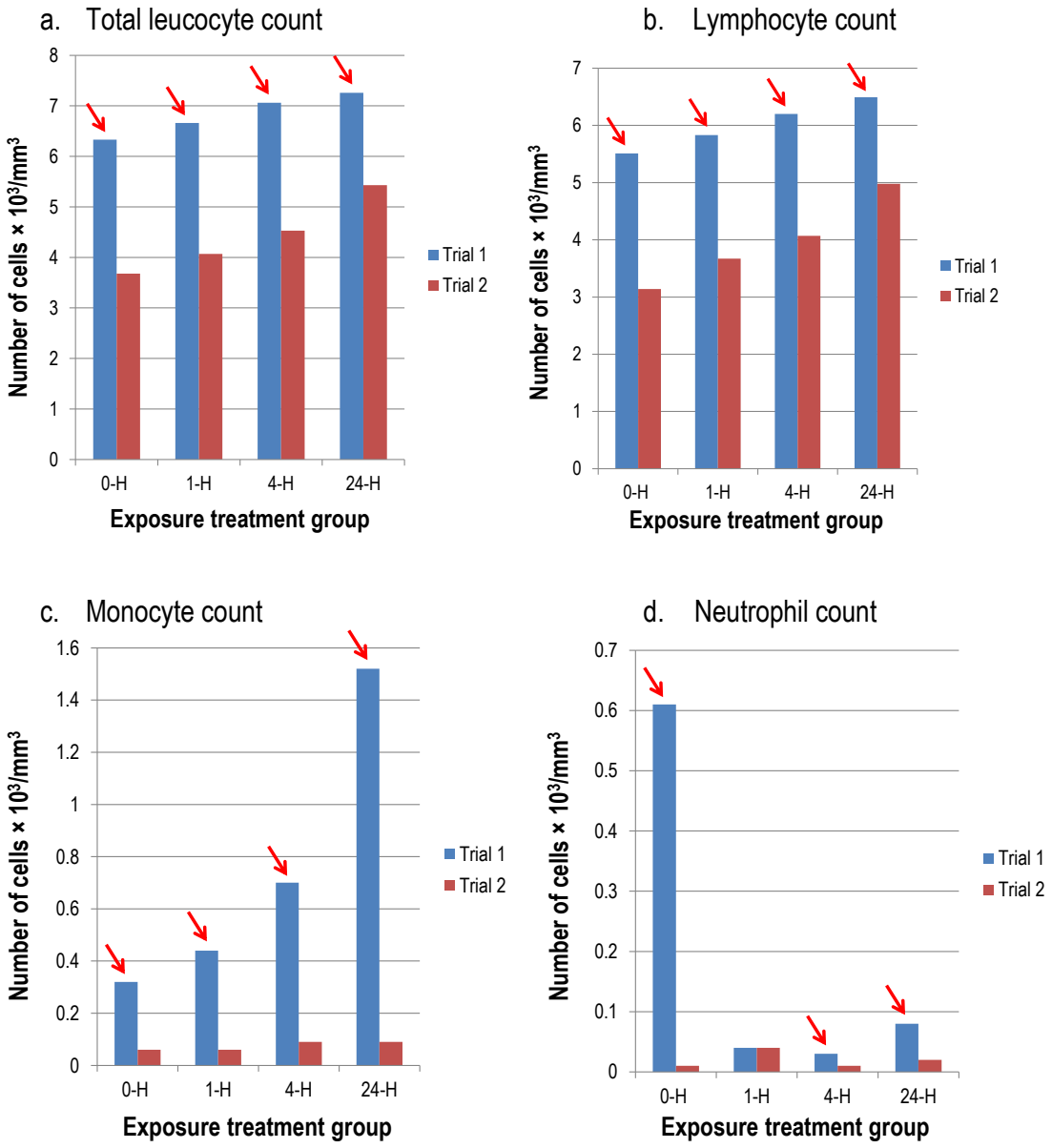
H = Hour; Exp. 1 = Experimental Trial 1; Exp. 2 = Experimental Trial 2

T-tests were performed to ascertain if the respective treatment groups of a particular leucocyte parameter of Experimental Trial 1 differed significantly from the equivalent treatment group in Experimental Trial 2. The t-tests revealed that most of the comparisons were highly significantly different at $\alpha = 0.05$ (Table 5.8). No significant differences could be demonstrated for the 1-hour ELF-EMF exposure treatment group of the neutrophils, and both of the 4-hour and 24-hour ELF-EMF exposure treatment groups of the basophils.

Table 5.8 **T-tests comparing the leucocyte parameters of Experimental Trials 1 and 2.**

Parameter	Treatment group	df	t	*P*-value
Total leucocytes	0-H	20	4.00	**0.0007**
	1-H	38	5.71	**< 00001**
	4-H	39	5.56	**< 0.001**
	24-H	41	3.52	**0.0011**
Lymphocytes	0-H	20	3.69	**0.0015**
	1-H	38	5.03	**< 0.001**
	4-H	39	5.10	**< 0.001**
	24-H	41	3.26	**0.0022**
Monocytes	0-H	36	7.09	**< 0.0001**
	1-H	30	7.08	**< 0.0001**
	4-H	29	4.98	**< 0.0001**
	24-H	25	2.46	**0.021**
Neutrophils	0-H	24	18.36	**< 0.0001**
	1-H	14	-0.47	0.650
	4-H	39	6.10	**< 0.0001**
	24-H	39	4.49	**< 00001**
Eosinophils	0-H	15	-5.07	**0.0001**
	1-H	38	7.66	**< 00001**
	4-H	39	3.64	**0.0007**
	24-H	41	3.25	**0.0023**
Basophils	0-H	15	-3.22	**0.0056**
	1-H	29	3.64	**0.0010**
	4-H	18	-0.02	0.980
	24-H	38	1.96	0.057

A visual perspective of the mean number of cells of the respective treatment groups of the two experimental trials showed several identifiable differences. For the four parameters total leucocytes, lymphocytes, monocytes and basophils; the mean number of cells was substantially greater in Experimental Trial 1 than those in Experimental Trial 2 (Figure 5.4). The mean number of eosinophils was also greater in Experimental Trial 1, but only for the ELF-EMF exposure treatment groups. The mean number of neutrophils also demonstrated similar results, except for the 1-hour exposure treatment group, where the two trials showed similar mean cell counts.



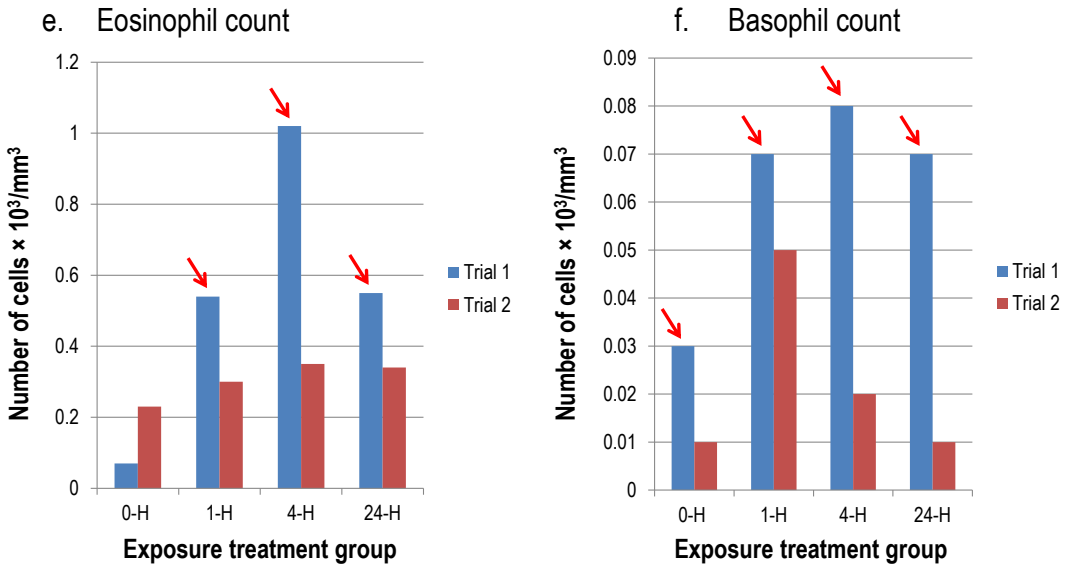


Figure 5.4 **Bar graphs of the comparison of Experimental Trial 1 and 2 mean values of the different leucocyte parameter treatments.**

5.5 Discussion

In contrast to human studies, several studies support the notion that ELF-EMF may influence leucocyte parameters. Similar to a number of previous studies in mice, this study showed an increase in the total leucocyte count after a short-term ELF-EMF exposure of one week, albeit not a significant increase. This increase was mainly represented by a non-significant increase in the number of lymphocytes. Other parameters that were affected by a one-week ELF-EMF exposure included a significant decrease in the number of neutrophils and basophils, while the number of eosinophils was significantly increased. In another study, exposure to ELF-EMF for one week also revealed an increase in the total leucocyte count in mice (de Kleijn et al. 2016). This increase was mainly represented by an increase in neutrophils, which was in contrast to what was found in this study. Similar to this study, Singh et al. (2012) also found that the number of leucocytes and lymphocytes was increased in mice exposed to ELF-EMF for six weeks, while the number of neutrophils were decreased. However, Hashish et al. (2008) demonstrated that the lymphocyte number decreased in mice after continuous exposure to ELF-EMF for 30 days. An opposing

outcome to these studies was the finding that no significant differences existed between control treatment groups and ELF-EMF exposure treatment groups for leucocyte parameters in mice after 40 days of exposure (Cicekcibasi et al. 2008). Long-term exposure to ELF-EMF; 12 weeks in this study and 15 weeks in a study conducted by de Kleijn et al. (2016), did not reveal any significant differences between the control treatment groups and the ELF-EMF exposure groups within a particular leucocyte parameter. De Kleijn et al. (2016) suggested that mice undergoing a type of adaptation to ELF-EMF could explain the absence of differences between treatments, within a particular leucocyte parameter, in the long-term ELF-EMF exposure experiment. Bonhomme-Faivre et al. (1998a) suggested that this adaption to ELF-EMF could be attributed to a recovery of the haemopoetic system.

In this study, immunological modifications were observed in leucocyte numbers in the 1-week experimental trial that were similar to those found by de Kleijn et al. (2016). De Kleijn et al. (2016) suggested that these changes may linked to stress-related parameters. In the short-term exposure ELF-EMF experiment conducted by de Kleijn et al. (2016) the stress-related parameter proopiomelanocortin (POMC) expression and plasma adrenocorticotropic hormone were significantly lower in the ELF-EMF exposed treatment group compared with unexposed control mice. De Kleijn et al. (2016) concluded that short-term ELF-EMF exposure may affect hypothalamic-pituitary-adrenal axis activation in mice and result in changes in stress hormone release, which in turn may explain changes in circulating leucocyte numbers and composition.

Noteworthy outcomes of this study were the significant differences between most of the 1-week treatments and the 12-week treatments, including the control treatments. The exceptions were the number of neutrophils exposed for one hour and number of basophils exposed for four and 24 hours. Overall, the cell numbers were lower in the 12-week experimental trial when compared to the 1-week experimental trial, which supports the results obtained by de Kleijn et al. (2016), who attributed the

lower cell numbers to batch variation. Having found similar results to de Kleijn et al. (2016), it is unlikely that batch variation alone can be responsible for the lowered cell numbers in the 12-week experimental trial. In a similar experiment with rats, (Szemerszky et al. 2010) did find a significant proopiomelanocortin elevation and depressive-like behaviour following a 6-week continuous exposure to ELF-EMF exposure. Other contributing factors explaining the differences between the results of the control treatment groups of the short-term and long-term experiments could be attributed to subclinical stress caused by confinement (Moberg 2000; Dhabhar 2014), as well as the animal age difference at sacrifice (Çetin et al. 2006). The age of the animals at sacrifice of the short-term experiment was 35 days, while the age of the animals of the long-term experiment was 112 days.

Chapter 6

Lymphocyte Cluster of Differentiation

6.1 Introduction

When cells differentiate, they undergo changes in gene expression. These changes are often expressed as changes in cell surface proteins. Many cell types express unique cell surface proteins. These proteins are used to characterise a cell's specific origin, its state of differentiation and its state of activation (Delves et al. 2017).

When investigating and identifying cell types; cell surface proteins are used as target molecules by applying the cluster of differentiation (CD) protocol. This protocol is applied particularly in immunophenotyping. In immunophenotyping these target cell surface proteins are referred to as CD markers and are used to associate cells with certain immune functions (Martini 2004). While the use of single CD markers to define cell populations is uncommon, the combination of CD markers allows for the identification of cell types with very specific definitions within the immune system. While only a fraction of the known CD markers has been thoroughly characterised, most of them have an important function, for example, CD4 and CD8 are critical in antigen recognition (Delves et al. 2017).

Blood cells are divided into three lineages, of which the lymphocyte lineage forms the foundation of the adaptive immune system. The lymphocyte lineage of white blood cells is formed in the bone marrow and comprise of T-lymphocytes and B-lymphocytes (Figure 6.1). T-lymphocytes undergo maturation in the thymus, while B-lymphocytes leave the bone marrow and migrate to peripheral lymphoid tissues, such as lymph nodes (Marieb and Hoehn 2016). T-lymphocytes play a central role in cell-mediated immunity, while B-lymphocytes function in the humoral immunity system of the adaptive immune system. Helper T-lymphocytes are required for almost all adaptive immune responses and are responsible for the activation of other cells such as B-lymphocytes and

macrophages. They facilitate B-lymphocytes to secrete antibodies and macrophages to destroy ingested microorganisms, but they also facilitate the activation of cytotoxic T-lymphocytes to destroy infected target cells (Martini 2004). Cytotoxic T-lymphocytes destroy certain cell types, including foreign cells, cancer cells, and virus infected cells.

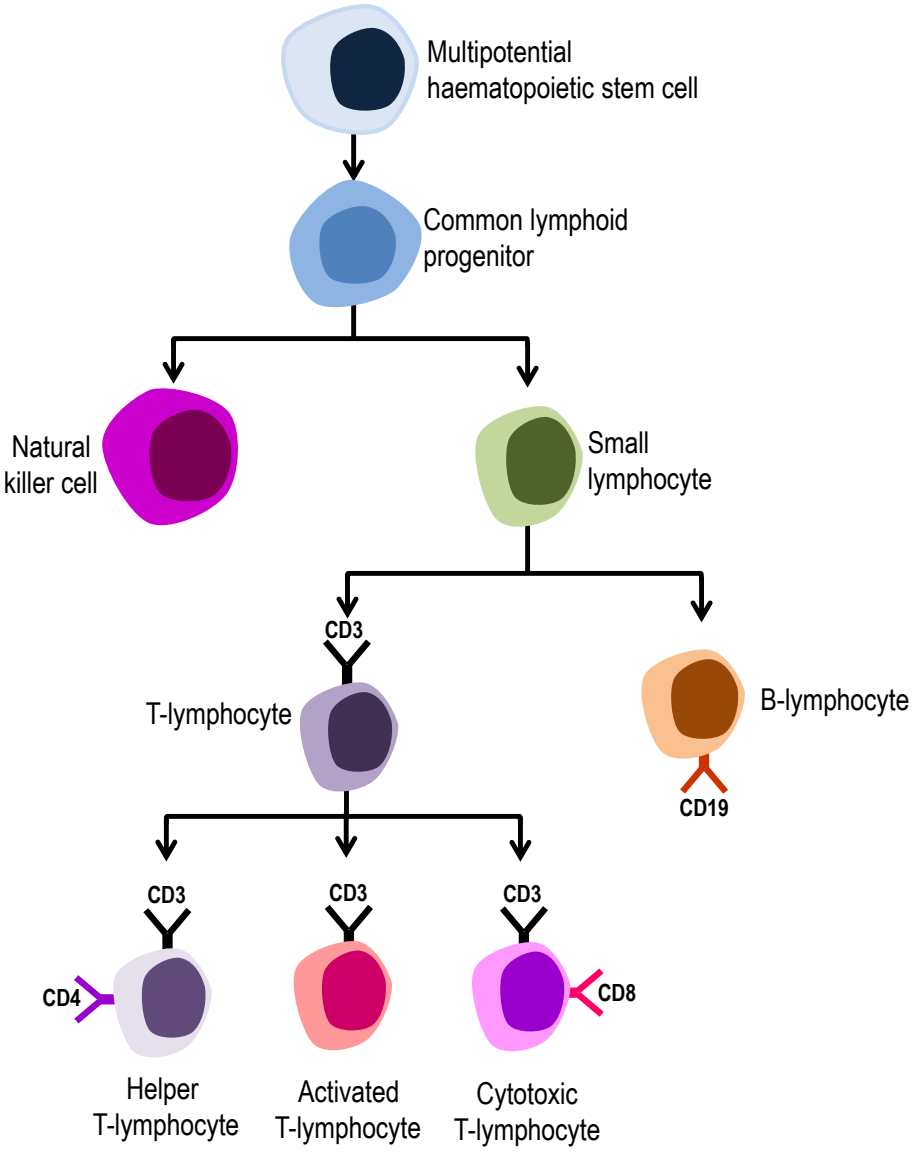


Figure 6.1 T- and B-Lymphocyte CD lineages.

The helper and cytotoxic T-lymphocyte populations are identified by using two CD markers in combination. The CD3 marker, which occurs on a number of different cell types, is used in combination with the CD4 marker to identify the helper T-lymphocyte population, and in combination with CD8 to identify the cytotoxic T-lymphocyte population. CD3 is a multimeric protein complex composed of four distinct polypeptide chains; epsilon (ε), gamma (γ), delta (δ) and zeta (ζ), that assemble and function as three pairs of dimers (εγ, εδ, ζζ) (Chetty and Gatter 1994; Kjer-Nielsen et al. 2004). The CD4 marker is a monomeric type I transmembrane glycoprotein, while the CD8 marker is a heterodimeric type I transmembrane glycoprotein comprising of an alpha (α) and a beta (β) polypeptide chain (Li et al. 2013). For the B-lymphocyte population, CD19 is a hallmark marker, which is a type I transmembrane glycoprotein (Wang et al. 2012).

There is a paucity of studies investigating the effects of ELF-EMF on lymphocyte cell populations using CD markers in animals. De Kleijn et al. (2016) demonstrated an increase in CD4 lymphocytes in mice exposed to ELF-EMF continuously for seven days. In contrast, Bonhomme-Faivre et al. (2003) found that the CD4 lymphocyte population decreased significantly in mice after continuous exposure to ELF-EMF in a long-term experiment of 109 days. In this experiment the total lymphocyte population, as well as the natural killer lymphocyte population, were also significantly lower when compared to the control treatment group. Hashish et al. (2008) also demonstrated decreased numbers of T- and B-lymphocytes in mice exposed continuously to ELF-EMF for 30 days.

The effects of ELF-EMF were studied on four CD markers in mice exposed for one week (Experimental Trial 1) and for twelve weeks (Experimental Trial 2). Three CD markers were used to enumerate three T-lymphocyte subpopulations. CD3 was used to identify the T-lymphocytes, CD4 the helper T-lymphocytes and CD8 the cytotoxic T-lymphocytes. CD19 was used to identify the B-lymphocyte population.

6.2 Experimental Trial 1: One-week exposure

Overall, a few distinguishing patterns emerged for the four treatment groups of the different CD parameters. The ranges of the mean percentages were, for the most, similar for each of the treatment groups within the respective CD parameters (Table 6.1). The exceptions were the ranges of the mean percentages of the 24-hour treatment groups of CD3, CD4 and CD8 parameters. These treatment groups displayed relatively narrow ranges compared to the other treatment groups; which could be attributed to the substantial drop in the maximum values of the mean percentages within these treatment groups. The mean percentages demonstrated a slight increase towards the higher ELF-EMF exposure times for all the CD parameters.

Most of the mean percentages of CD3, CD4 and CD8 parameters fell within the range of the control treatment group; except for the maximum percentages of the 1-hour and 4-hour treatment groups that were greater than the maximum percentage of the control treatment group. Similarly, for the CD19 parameter, most of the mean percentages fell within the range of the control treatment group; except for the minimum percentages of the 1-hour and 24-hour treatment groups that fell below the minimum percentage of the control treatment group.

Table 6.1 Summary statistics of the CD parameters of Experimental Trial 1.

Variable	Treatment group	Minimum	Maximum	Range	Mean	Standard deviation
CD3	0-H (control)	7.40	79.00	71.60	26.06	17.49
	1-H	12.10	84.00	71.90	42.13	17.91
	4-H	14.20	92.00	77.80	38.26	16.64
	24-H	14.30	55.50	41.20	34.68	9.11
CD4	0-H (control)	5.10	57.00	51.90	19.42	13.40
	1-H	8.50	63.00	54.50	31.40	13.20
	4-H	10.60	68.00	57.40	28.08	11.98
	24-H	11.50	44.80	33.30	26.78	6.98
CD8	0-H (control)	1.30	20.00	18.70	6.70	4.21
	1-H	5.00	41.00	36.00	12.50	7.44
	4-H	2.40	29.00	26.60	10.28	6.35
	24-H	1.70	13.90	12.20	8.55	3.04
CD19	0-H (control)	2.00	59.00	57.00	18.11	15.80
	1-H	1.80	49.00	47.20	21.57	15.05
	4-H	3.20	44.20	41.00	21.42	12.06
	24-H	1.10	43.80	42.70	23.23	11.86

H = hour

A bar graph was constructed of the mean percentages of all the treatments of the different CD parameters to provide a visual perspective of the percentages of the respective treatments within each parameter. For all the CD parameters, the ELF-EMF exposure treatment groups displayed percentages that were greater than that of the control treatment group (0-hour control treatment group) (Figure 6.2). However, the mean percentages of the different treatment groups of parameter CD19 were, overall, rather similar. For all four CD parameters, the 1-hour treatment group demonstrated the highest mean percentages.

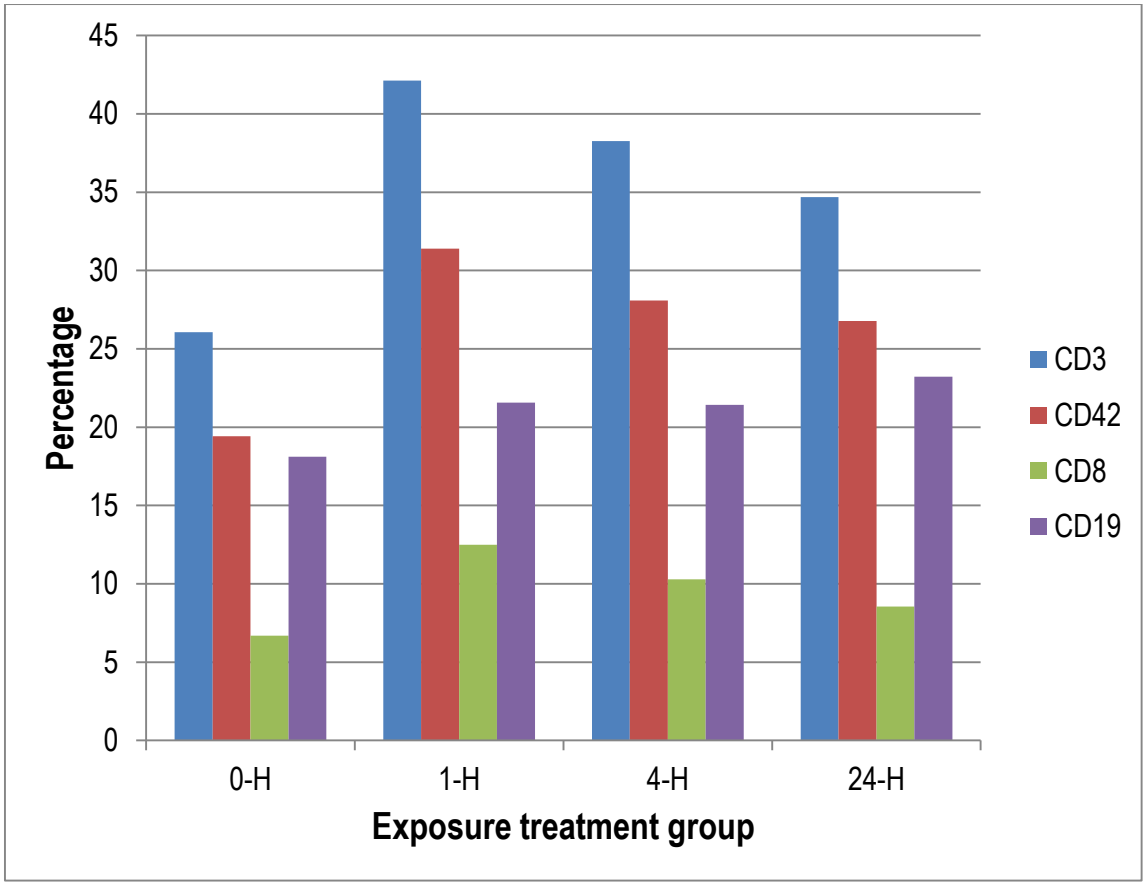


Figure 6.2 **Bar graph showing the mean percentages of the different CD parameter treatments for Experimental Trial 1.**

ANOVA tests were performed to ascertain if significant differences existed between the different treatment groups within the different CD parameters. Highly significant differences were identified for the three parameters CD3, CD4 and CD8 at $\alpha = 0.05$ (Table 6.2). The ANOVA test performed on the treatment groups of CD19 parameter did not reveal significant differences.

Table 6.2 ANOVA tests performed on the CD parameters of Experimental Trial 1.

Parameter	Source	Sum of squares	Degrees of freedom	Mean square	*F*	*P*-value
CD3	Between groups	3,529.97	3.00	1,176.66	5.01	**< 0.001**
	Within groups	16,917.62	72.00	234.97		
	Total	27,206.48	99.00			
CD4	Between groups	1,920.46	3.00	640.15	5.00	**< 0.001**
	Within groups	9,210.66	72.00	127.93		
	Total	15,024.82	99.00			
CD8	Between groups	452.25	3.00	150.75	4.89	**< 0.001**
	Within groups	2,221.91	72.00	30.86		
	Total	3,397.29	99.00			
CD19	Between groups	345.70	3.00	115.23	0.68	0.57
	Within groups	12,272.28	72.00	170.45		
	Total	18,649.55	99.00			

H = hour

Tukey HSD tests were performed on CD3, CD4 and CD8 parameters to determine which of the different treatment groups differed from one another at $\alpha = 0.05$. The Tukey HSD tests revealed that the 1-hour and 4-hour treatment groups differed from the control treatment group for CD3 and CD4 parameters (Table 6.3). For CD8 parameter only the 1-hour treatment group differed from the control treatment group.

Table 6.3 Results of Tukey HSD tests for CD3, CD4 and CD8.

CD3				
TG	**0-H**	**1-H**	**4-H**	**24-H**
0-H				
1-H	**S**			
4-H	**S**	NS		
24-H	NS	NS	NS	

CD4				
TG	**0-H**	**1-H**	**4-H**	**24-H**
0-H				
1-H	**S**			
4-H	**S**	NS		
24-H	NS	NS	NS	

CD8				
TG	**0-H**	**1-H**	**4-H**	**24-H**
0-H				
1-H	**S**			
4-H	NS	NS		
24-H	NS	NS	NS	

S = significant; NS = non-significant; TG = treatment group; 0-H = control, 1-H = 1 hour, 4-H = 4 hours, 24-H = 24 hours

6.3 Experimental Trial 2: Twelve-week exposure

Overall, a few distinguishing patterns emerged that were clearly different when compared to Experimental Trial 1 for the four treatment groups of the different CD parameters. The ranges of the mean percentages were for all the treatment groups within the respective CD parameters smaller than those of Experimental Trial 1 (Table 6.4). The smaller ranges of the mean percentages were reflected by their increased minimum values and their decreased maximum values. The mean percentages recorded for CD3, CD4 and CD8 were greater than those recorded in Experimental Trial 1 for the control treatment groups, the 4-hour and the 24-hour treatment groups. The mean percentages of the 1-hour treatment group of these CD parameters were less that those recorded for Experimental Trial 1. In contrast, the mean percentages of all the treatment groups of CD19 were less than those recorded for Experimental Trial 1.

Table 6.4 Summary statistics of cluster of CD of Experimental Trial 2.

Variable	Treatment group	Minimum	Maximum	Range	Mean	Standard deviation
CD3	0-H (control)	14.09	58.88	44.79	39.47	15.79
	1-H	15.09	69.96	54.87	39.27	13.62
	4-H	17.06	71.45	54.39	47.13	15.02
	24-H	13.66	70.71	57.05	43.83	15.99
CD4	0-H (control)	9.78	47.32	37.54	29.89	12.53
	1-H	9.70	54.05	44.35	29.27	11.12
	4-H	13.09	53.85	40.76	36.06	12.43
	24-H	6.36	52.65	46.29	33.50	13.07
CD8	0-H (control)	3.98	18.28	14.30	10.57	3.65
	1-H	3.79	18.47	14.68	11.04	3.39
	4-H	4.26	20.83	16.57	12.24	4.59
	24-H	4.96	26.00	21.04	12.21	4.65
CD19	0-H (control)	0.66	32.43	31.77	14.54	9.99
	1-H	1.38	30.00	28.62	12.07	6.27
	4-H	4.07	25.20	21.13	15.00	5.35
	24-H	3.25	37.12	33.87	16.99	9.26

H = hour

A bar graph showing the mean percentages of all the treatments of the different CD parameters provides a visual perspective of the percentages of the respective treatments within each parameter. For the three CD parameters, CD3, CD4 and CD8, the 4-hour treatment groups demonstrated the highest mean percentages. This was in contrast to what was found for Experimental Trial 1, where the highest values were found for the 1-hour treatment group (Figure 6.3). The highest mean percentage for CD19 was recorded for the 24-hour treatment group.

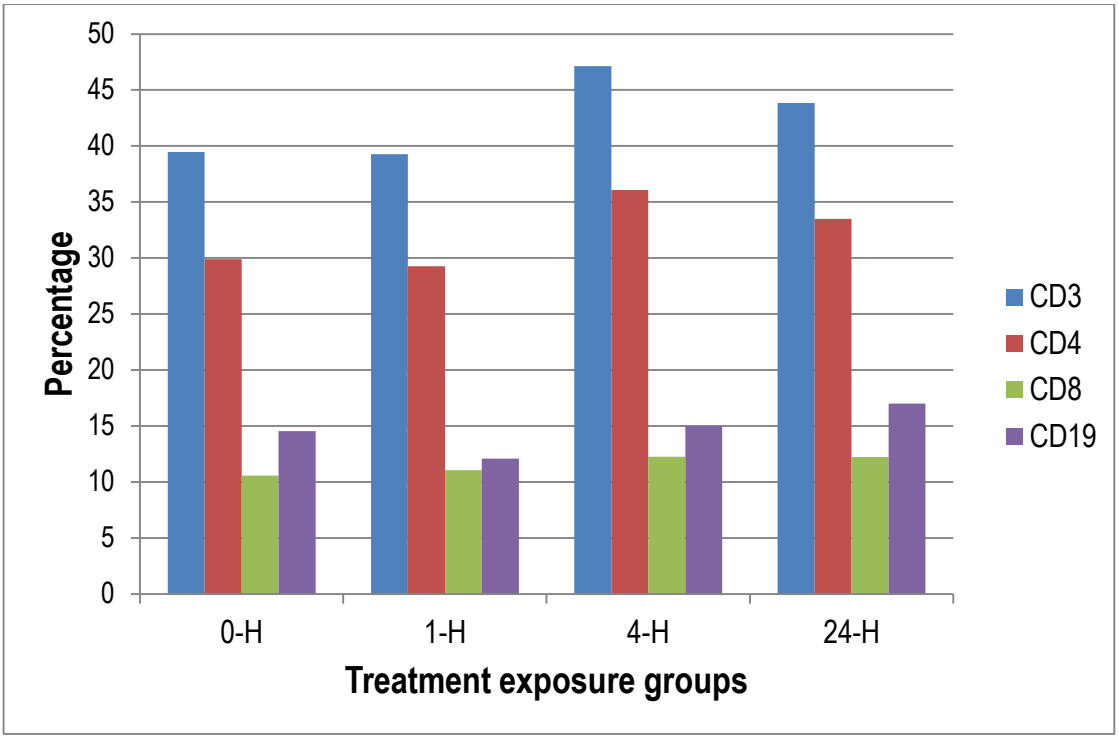


Figure 6.3 **Bar graph showing the mean percentages of the different CD parameter treatments for Experimental Trial 2.**

ANOVA tests were performed to ascertain if significant differences existed between the different treatment groups within the different CD parameters. No significant differences were found for all the CD parameters at $\alpha = 0.05$ (Table 6.5).

Table 6.5 **ANOVA tests performed on the CD parameters of Experimental Trial 2.**

Parameter	Source	Sum of squares	Degrees of freedom	Mean square	*F*	*P*-value
CD3	Between groups	951.22	3.00	317.07	1.40	0.25
	Within groups	19,768.25	87.00	227.22		
	Total	20,719.46	90.00			
CD4	Between groups	702.64	3.00	234.21	1.55	0.21
	Within groups	13,141.35	87.00	151.05		
	Total	13,843.98	90.00			

Parameter	Source	Sum of squares	Degrees of freedom	Mean square	*F*	*P*-value
CD8	Between groups	44.84	3.00	14.95	0.87	0.46
	Within groups	1,491.93	87.00	17.15		
	Total	1,536.78	90.00			
CD19	Between groups	3.6.78	3.00	102.26	1.69	0.18
	Within groups	5,257.86	87.00	60.44		
	Total	5,564.64	90.00			

H = hour

6.4 Comparison of Experimental Trial 1 and Experimental Trial 2

When the ranges of the mean percentages of the respective treatment groups of the different CD parameters of the two experimental trials were compared, some distinguishing patterns could be identified. The ranges were, for the most, larger for Experimental Trial 1 than for Experimental Trial 2 (Table 6.6). The exceptions were the 24-hour treatment groups of CD3, CD4 and CD8, which displayed relatively narrow ranges of the mean percentages. It was difficult to identify distinguishing patterns when comparing the mean percentages of the treatment groups of the respective CD parameters. However, it was noted that for CD3, CD4 and CD8, the differences in the mean percentages of Experimental Trial 1 were greater for the 1-hour treatment groups when the two trials were compared, while the mean percentages of the other treatment groups of Experimental Trial 1 were all smaller than those of Experimental Trial 2. For the other treatment groups of CD3, CD4 and CD8, the mean percentages of Experimental Trial 2 were all greater than Experimental Trial 1. For CD19, all the mean percentages of Experimental Trial 1 were greater than Experimental Trial 2.

Table 6.6 Comparison of ranges and means between Experimental Trials 1 and 2.

Variable	Treatment group	Range 1-week	Mean 1-week	Range 12-week	Mean 12-week	Difference in ranges	Difference in means
CD3	0-H (control)	71.60	26.06	44.79	39.47	26.81	-13.41
	1-H	71.90	42.13	54.87	39.27	17.03	2.86
	4-H	77.80	38.26	54.39	47.13	23.41	-8.87
	24-H	41.20	34.68	57.05	43.83	-15.85	-9.15
CD4	0-H (control)	5.90	19.42	37.54	29.89	-31.64	-10.47
	1-H	54.50	31.40	44.35	29.27	10.15	2.13
	4-H	57.40	28.08	40.76	36.06	16.64	-7.98
	24-H	33.30	26.78	46.29	33.50	-12.99	-6.72
CD8	0-H (control)	18.70	6.70	14.30	10.57	4.40	-3.87
	1-H	36.00	12.50	14.68	11.04	21.32	1.46
	4-H	26.60	10.28	16.57	12.24	10.03	-1.96
	24-H	12.20	8.55	21.04	12.21	-8.84	-3.66
CD19	0-H (control)	57.00	18.11	31.77	14.54	25.23	3.57
	1-H	47.20	21.57	28.62	12.07	18.58	9.50
	4-H	41.00	21.42	21.13	15.00	19.87	6.42
	24-H	42.70	23.23	33.87	16.99	8.83	6.24

H = hour

T-tests were performed to ascertain if the respective treatment groups of a CD parameter of Experimental Trial 1 differed significantly from the equivalent treatment group in Experimental Trial 2. The t-tests revealed that 63% of the comparisons demonstrated significant or highly significant differences at $\alpha = 0.05$ (Table 6.7). Two distinguishing patterns were noted. For CD3, CD4 and CD8, all the control treatment groups demonstrated significant differences between the two trials. For the 24-hour treatment groups, the t-tests revealed significant differences for all the CD parameters.

Table 6.7 T-tests comparing the CD parameters of Experimental Trials 1 and 2.

Parameter	Treatment group	df	t	*P*-value
CD3	0-H	37	-2.58	**0.01**
	1-H	45	0.64	0.53
	4-H	47	-1.94	0.05
	24-H	38	-2.49	**0.02**
CD4	0-H	36	-2.58	**0.01**
	1-H	47	0.62	0.54
	4-H	47	-2.28	**0.03**
	24-H	37	-2.27	**0.03**
CD8	0-H	38	-3.12	**< 0.001**
	1-H	32	0.90	0.37
	4-H	44	-1.23	0.22
	24-H	31	4.32	**< 0.001**
CD19	0-H	40	0.89	0.38
	1-H	32	2.91	**< 0.001**
	4-H	33	2.44	**0.02**
	24-H	45	2.01	**0.04**

A visual perspective of the mean percentages of the respective exposure treatment groups of the two experimental trials shows the major differences. For the CD parameters CD3, CD4 and CD8, three of the treatments groups demonstrated higher mean percentages in Trial 2 than Experimental Trial 1 (Figure 6.3). For CD19 all the mean percentages of Experimental Trial 1 were greater than those of Experimental Trial 2.

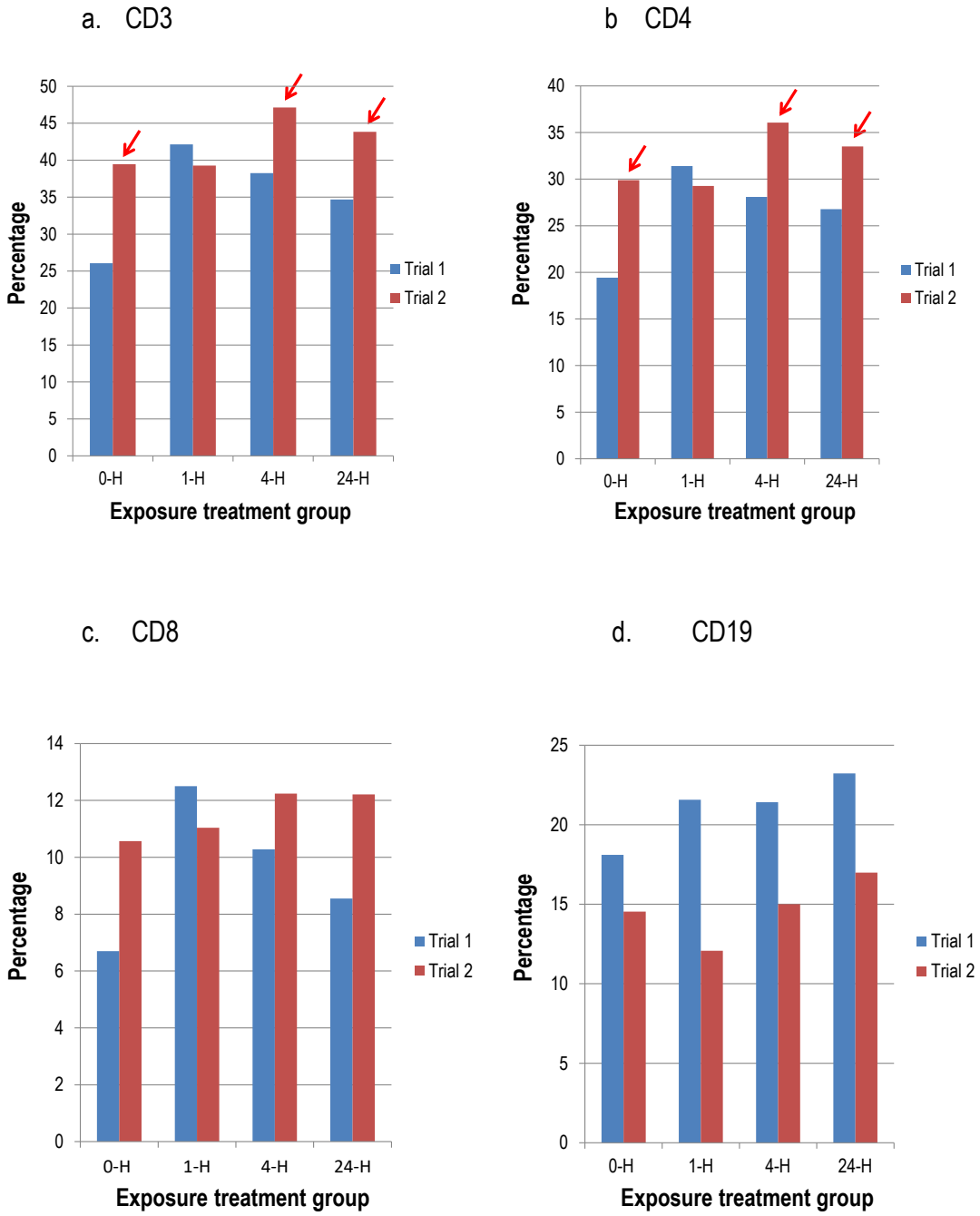


Figure 6.4 **Bar graph of the comparison of Experimental Trial 1 and 2 mean percentages of the different CD parameter treatments.**

6.5 Discussion

The lymphocyte subsets as identified by the four CD parameters CD3, CD4, CD8 and CD19 demonstrated interesting differences between the T-lymphocyte populations and the B-lymphocyte populations. Mice that were ELF-EMF exposed for one hour in the one-week experimental trial demonstrated a substantial increase in the T-lymphocyte parameters CD3, CD4 and CD8, when compared to the other exposure treatment groups. Although T-lymphocyte subsets were greater than the control treatment groups in the longer exposure treatment groups, they were less than the 1-hour treatment groups, with the 24-hour treatment groups approaching the values of the control treatment groups. De Kleijn et al. (2016) also found in a one-week exposure trial that CD3 and CD4 lymphocyte subsets were significantly greater than the control treatment groups. In contrast, in the 12-week trial of this study, the T-lymphocyte parameters CD3, CD4 and CD8 were substantially increased for all the exposure treatment groups, except for the 1-hour treatment groups. In a similar trial of 15 weeks, de Kleijn et al. (2016) found contrasting results, where no significant differences could be established for all the treatment groups of the CD3 and CD4 lymphocyte subsets. In a study conducted by Bonhomme-Faivre et al. (2003), the CD4 lymphocyte subset was substantially lower than the control group after 109 days of continuous exposure to ELF-EMF.

The numeration of the CD19 B-lymphocyte subpopulations revealed conflicting patterns to what was found for the T-lymphocyte populations. In the one-week experimental trial, the CD19 lymphocytes were increased in all ELF-EMF exposure treatment groups. When the 12-week experimental trial was considered, all the treatment groups were substantially lower when compared to the one-week experimental trial. However, only the one-hour treatment group showed values that were less than the control treatment group. It appeared as if Bonhomme-Faivre et al. (2003) found similar outcomes for the CD19 lymphocyte subset. The CD19 lymphocytes of the ELF-EMF exposure treatment groups in the one-week experimental trials were all increased. Similarly, to the results of this study

de Kleijn et al. (2016) also found that the numbers of CD19 lymphocytes of all treatment groups were less in the 15-week trial when compared to the one-week trial. However, in contrast to this study, the one-hour treatment group in the 15-week trial demonstrated greater numbers that the control group. In a medium-term continuous exposure experiment, Hashish et al. (2008) identified decreased levels of B-lymphocytes in ELF-EMF exposed mice.

This study suggests that the cell-mediated immune system, as well as the humoral immune system is affected differently when mice are exposed to ELF-EMF. In the short-term, mild exposure to ELF-EMF (one hour per day for one week) may lead to the stimulation of the cell-mediated immune system. In contrast, when mice are exposed to ELF-EMF for a relatively long period, the cell-mediated immune system appears to be stimulated by all periods of ELF-EMF exposure. The B-lymphocytes of the humoral immune system, on the other hand, are stimulated in mice by any ELF-EMF exposure in the short-term. In the long-term, all mice, whether exposed to ELF-EMF or not, respond with lowered B-lymphocyte populations, thereby compromising their adaptive immune system.

Chapter 7

Discussion and Conclusions

7.1 Introduction

Human exposure to extremely low frequency electromagnetic fields (ELF-EMF) is part of modern life. These electromagnetic fields are generally frequencies < 300 Hz (Otto and von Mühlendahl 2007; Ortega-Garcia et al. 2009; Redlarski et al. 2015; Zhang et al. 2016). During the 20th century, modern life has resulted in a steadily increasing exposure to ELF-EMF caused by devises that are human-made (D'Angelo et al. 2015).

Both at home and at work, humans are exposed to a complex mixture of ELF-EMF, arising from artificial sources generating and transmitting electricity. These sources include domestic appliances, industrial equipment, telecommunications and broadcasting. The introduction of residential and industrial use of electricity for power, heating, and lighting, has brought about far greater and increasing exposures over the last 120 years (Ahlbom et al. 2001).

The ever-increasing environmental exposure to ELF-EMF is of concern, mainly because of the potential for these electromagnetic fields to cause adverse health effects. Biological effects of ELF-EMF exposures have been noted frequently (Bräscher et al. 2017), although the implication for specific health effects is not that clear (Simkó and Mattsson 2004). Several epidemiological studies have shown a possible association between human ELF-EMF exposure and increased incidence of childhood leukaemia, brain tumours or neurodegenerative diseases, breast cancer, and cardiovascular disease (Bouwens et al. 2001; D'Angelo et al. 2015). However, some studies have produced contrasting and beneficial results; for example, accelerated wound (Callaghan et al. 2008; Goudarzi et al. 2010) and bone healing (Bassett 1965; 1967; Dimitriou and Babis 2007; Pall 2013),

as well as an enhanced response of macrophages to bacterial challenges in humans (Akan et al. 2010). In coccidian-infected chickens, a decreased severity of intestinal lesions was also observed (Elmusharaf et al. 2007).

The basic interaction mechanism(s) between ELF-EMF exposure and living matter is still largely unknown. Hypotheses have been suggested, although none is convincingly supported by experimental data (Simkó and Mattsson 2004). Various cellular components, processes, and systems can be affected by ELF-EMF exposure, therefore it has been suggested that ELF-EMF exposure results in signal transduction pathways and modulation of oxygen radicals (Liburdy et al. 1993; Rollwitz et al. 2004; Simkó and Mattsson 2004; Markov 2007; Frahm et al. 2010). Cuppen et al. (2007) also suggested that ELF-EMF exposure causes stress at the cellular level and that this leads to the production of cytokines and consequently several biological responses, including immune responses. A similar hypothesis, put forward by de Kleijn et al. (2016), suggested that ELF-EMF exposure may affect stress regulation and, as a result, will affect number and activation of leukocytes.

Animal models are extensively used to provide insight into the mechanisms underlying many human diseases. Although larger species, such as dogs, pigs and non-human primates, are more closely related to humans than mice; working with these large animals is extremely expensive and is fraught with ethical concerns. Thus, mouse models are still used extensively as a means to elucidate mechanisms of human pathology (Justice and Dhillon 2016). This study was thus undertaken to elucidate the effects of ELF-EMF on red blood cells, thrombocytes, white blood cells and lymphocyte cluster of differentiation markers in the BALB/c N_1H mouse strain.

7.2 Effects of ELF-EMF exposure

Contrary to the expectation for this project, not all blood parameters were affected by short-term ELF-EMF exposure. In the short-term exposure experiment, a few noteworthy outcomes could be identified. ELF-EMF did not have a significant ($p > 0.05$) effect on the erythrocyte population and the related parameters haemoglobin concentration and haematocrit. A similar outcome was demonstrated by the thrombocyte population. Similarly, the overall leucocyte population also was not affected by ELF-EMF exposure. However, when investigating the different leucocyte cell types, significant changes were noted in the number of granulocytes ($p < 0.0001$), whereas the agranulocytes did not reveal any significant differences in number ($p > 0.05$). The number of eosinophils and basophils was elevated, while the number of neutrophils was suppressed. The T-lymphocyte cluster of differentiation subtypes, CD3, CD4 and CD8 populations demonstrated significant increases in number, particularly in the one-hour per day ELF-EMF exposure group ($p < 0.001$). The B-lymphocyte cluster of differentiation subtype CD19, did not show any differences when compared to the control group ($p = 0.57$).

Similarly, to the short-term exposure experiment, not all blood parameters were affected by long-term ELF-EMF exposure. When considering the long-term exposure experiment an unexpected outcome was encountered for the control treatment groups. The control groups were, for the most, highly significantly different when compared to the short-term exposure experiment ($p < 0.001$). In this study, the control groups showed suppressed numbers of total leucocytes, lymphocytes, monocytes, neutrophils and basophils, while the eosinophils were significantly elevated when compared to the short-term experiment control groups. The control groups of the T-lymphocyte cluster of differentiation subtypes, CD3, CD4 and CD8 populations, were also elevated when compared to the short-term experiment. These data indicated that long-term confinement resulted in other physiological changes, irrespective whether the animals were exposed to ELF-EMF or not.

Because the animals in this study were considered to be specifically pathogen free and because exposure to microbes during the experiments was limited and equal in all treatment groups, it was concluded that illness due to exposure to pathogens could not be responsible for the difference. Confinement, by its nature, restricts behavioural options for the animals and it is also known that animals confined in cages for a lengthy period are more vulnerable to subtle effects of subclinical stress, which could have contributed to these results (Moberg 2000; Dhabhar 2014). Studies in rats indicated that ELF-EMF exposure enhanced the anxiety-like behaviour in the confined animals (Szemerszky et al. 2010). A further contributing factor to the difference in the results of the control groups of the short-term and long-term experiments could be attributed to the difference in age of the animals (Çetin et al. 2006); 35 days old in the short-term and 112 days in the long-term experiment.

When considering the effect of ELF-EMF in the long-term experiment, no significant differences could be established between the control treatment groups and the exposure treatment groups for all the parameters. De Kleijn et al. (2016) attributed these equivalent numbers to be as a result of long-term exposed mice undergoing adaptation to ELF-EMF. Bonhomme-Faivre and colleagues (1998a) also showed that mice exposed to ELF-EMF demonstrated recovery of the haemopoetic system even after 350 days of exposure. Although the control treatment group and exposure treatment groups of the different parameters did not differ from one another, it was noticeable that the four-hour per day ELF-EMF exposure resulted in an elevation of the number of red blood cells, haemoglobin concentration and haematocrit. Similarly, the basophils were also elevated in the one-hour per day ELF-EMF exposure group.

The debate continues about the consequences of ELF-EMF exposure to human health. Little evidence exists that ELF-EMF exposure affects blood cell parameters (Selmaoui et al. 1996; Bonhomme-Faivre, et al. 1998b; Dasdag et al. 2002; Ichinose et al. 2004; Touitou et al. 2013). A

number of epidemiologic studies appear to support the hypothesis that prolonged exposure to ELF-EMF may be associated with increased risk of childhood cancer, particularly lymphatic leukaemia, lymphomas, and brain tumours (Dasdag et al. 2002). In contrast, the results of this study support the notion that ELF-EMF exposure, in the short-term, could cause haematological (de Kleijn et al. 2016) and immunological modifications, albeit in mice. In the long-term, ELF-EMF exposure in mice remains difficult to interpret, however, indications suggest that recovery from the effects of ELF-EMF is possible.

7.3 Concluding remarks

The unexpected results of this study revealed that many gaps still exist in the understanding of the underlying effects of ELF-EMF exposure on living tissue, particularly blood parameters. In mice the activation of the immune response could be studied on bacterially infected animals. The role of ELF-EMF exposure on reactive oxygen species and cellular inflammation remain important questions to be answered. Another important aspect that requires investigation is the role of confinement of animals on the results of experiments. Finally, how the effects of ELF-EMF exposure in mice relate to ELF-EMF exposure humans, require further elucidation.

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Ethical Clearance

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Dear Ms Wiese

ANIMAL EXPERIMENT NR 20/08
PROJECT TITLE: "IMMUNE STIMULATION IN MICE (MUS. MUSCULUS) WITH EXPOSURE TO EXTREMELY LOW FREQUENCY (ELF) ELECTROMAGNETIC FIELDS (EMF)"

You are hereby kindly informed that the Interfaculty Animal Ethics Committee approved the following amendments to the above study at the meeting held on 27 October 2011:

a) *Change specie name in project title to Mus. musculus*
b) *Extension of approval to 2011*
c) *Change number of animals to 520*

Kindly take note of the following:

1. *Fully completed and signed applications have to be submitted electronically to StraussHS@ufs.ac.za and a hard copy has to be submitted too.*
2. *A signed progress report with regard to the above study has to be submitted electronically to StraussHS@ufs.ac.za while a hard copy has to be submitted to Ms H Strauss, Room D115, Francois Retief building, Faculty of Health Sciences. A report has to be submitted when animals are physically involved and after completion of the study. Guidelines with regard to progress reports are available from the secretary and on the network i:/algemeen/navorsing-research/etiekkomitees.doc.*
3. *Researchers that plan to make use of the Animal Experimentation Unit must request a quotation from the Head, Mr Seb Lamprecht*
4. *Contract research: Fifty (50%) of the quoted amount is payable when you receive the letter of approval.*

Regards

.....
CHAIR:
INTERFACULTY ANIMAL ETHICS COMMITTEE

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